Rec'd PCT/PTO 18 FEB 2005
PCT/[redacted] 03 01323
18 DECEMBER 2003 18-12-03

RECEIVED
28 JAN 2004
WIPO PCT

PA 1059297

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE

United States Patent and Trademark Office

September 03, 2003

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE UNDER 35 USC 111.

APPLICATION NUMBER: *60/404,922*
FILING DATE: *August 22, 2002*

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

N. WILLIAMS
Certifying Officer

BEST AVAILABLE COPY

PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION under 37 CFR 1.53(b)(2)

Docket Number	11402-1	Type a Plus Sign (+) Inside this Box -->	+

INVENTOR(S)/APPLICANT(S)

LAST NAME	FIRST NAME	Middle Initial	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
DOUGLAS	Susan		39 Stevens Road, RR1 Boutilier's Point, Nova Scotia, B0J 1G0, Canada
GALLANT	Jeffrey		10 Willowdale Terrace, Halifax, Nova Scotia, B3P 1S2, Canada

TITLE OF THE INVENTION (280 CHARACTERS MAX.)

A Genomic Approach To Identification Of Novel Broad-spectrum Antimicrobial Peptides From Bony Fish

CORRESPONDENCE ADDRESS

J. Wayne Anderson
National Research Council of Canada
Intellectual Property Services Office, EG-10, Bldg. M-58
Montreal Road, Ottawa, Ontario, Canada K1A 0R6

STATE	Ontario	ZIP CODE	K1A 0R6	COUNTRY	Canada

ENCLOSED APPLICATION PARTS (*Check all that Apply*)

X	*Specification*	*Number of pages*	49			*Small Entity Statement*	
X	*Drawing(s)*	*Number of Sheets*	23			*Other (specify)*	

METHOD OF PAYMENT (*Check One*)

	A check or money order is enclosed to cover the Provisional filing fees	*Provisional Filing Fee Amount ($)*	$ 160.00
X	*The Commissioner is hereby authorized to charge filing fees and any deficiency in the filing fees and credit to our Mastercard Account Form PTO-2038 is attached*		
X	*We hereby authorize you to deduct any deficiency of the fee stated above or credit to this amount to our Deposit Account 14-0429.*		

The invention was made by an agency of the United States Government or under contract with an agency of the United States Government. X No

Date: 20 August 2002

Respectfully submitted,

J. Wayne Anderson
Patent Agent for Applicant
Regn No. 28,158

Encl.
:ech

_____ Additional inventors are being named a separately numbered sheets attached hereto.

PROVISIONAL APPLICATION FILING ONLY

JC806 U.S. PTO 08/22/02
11017 U.S. PTO 60/404922 08/22/02

A Genomic Approach to Identification of Novel Broad-spectrum Antimicrobial Peptides From Bony Fish

BACKGROUND OF THE INVENTION

Antimicrobial peptides have been isolated from a wide variety of plants and animals, and play an important role in defense against microbial invasion. They fall into three main classes based on secondary structure and amino acid sequence similarities: α-helical structures, highly disulphide-bonded (cysteine-rich) β-sheets and those with a high percentage of proline or glycine residues. Most molecules are amphiphilic and contain both cationic and hydrophobic surfaces, enabling them to insert into biological membranes. Although the primary mode of action of antimicrobial peptides has been described as lysis of pathogens, they have also been reported to exert a number of other effects such as mediating inflammation and modulating the immune response (for review, see Hancock and Lehrer 1998).

A small number of natural antimicrobial peptides have been isolated from teleosts including the pleurocidin, from the skin of winter flounder (Cole, Weis et al. 1997), pardaxin from Red Sea Moses sole (Oren and Shai 1996), misgurnin from loach (Park, Lee et al. 1997), HFA-1 from hagfish (Hwang, Seo et al. 1999), piscidins from hybrid striped bass eosinophilic granule cells (Silphaduang and Noga 2001), moronecidins from hybrid striped bass (Lauth, Shike et al. 2002), parasin, a cleavage product of histone 2A from catfish (Park, Park et al. 1998) and some uncharacterized mucous secretions from carp (LeMaitre, Orange et al. 1996) and trout (Smith, Fernandes et al. 2000). In addition, a cationic steroidal antibiotic, squalamine, has been isolated from the shark, *Squalus acanthias* (Moore, Wehrli et al. 1993). Pleurocidin and parasin form amphiphathic structures, whereas the other compounds isolated from fish are structurally unrelated.

Cysteine-rich antimicrobial peptides of the defensin family have been detected in the fat body of insects and the hemolymph of molluscs and crustaceans. They have also been isolated from various epithelia of mammals as well as circulating cells such as neutrophils and macrophages. Recently, small cysteine-rich peptides exhibiting antimicrobial activity against various fungi, Gram positive and Gram negative bacteria

have been isolated from blood ultrafiltrate (Krause, Neitz et al. 2000), the human urinary tract (Park, Valore et al. 2001), and the gill of bacterially challenged hybrid striped bass (Shike et al. 2002). These peptides, referred to as hepcidin or LEAP-1 (liver-expressed antimicrobial peptide), have been proposed to be the vertebrate counterpart of insect peptides induced in the fat body in response to infection (Park, Valore et al. 2001).

SUMMARY OF THE INVENTION

According to one aspect of the invention the nucleotide and deduced amino acid sequences of 14 hepcidin-like peptides isolated from three diverse species of fish, winter flounder, Atlantic halibut and Atlantic salmon, are provided.

According to another aspect of the invention, the nucleotide and deduced amino acid sequences of 23 pleurocidins, isolated from different fish species is also provided.

According to yet another aspect of the invention, the amino acid sequences of pleurocidin-like peptides with confirmed antibacterial and antifungal activities, as well as the process for predicting said activities from genomic and expressed nucleotide sequences, are provided.

According to yet another aspect of the invention, a process is provided for the expression of genes encoding such peptides during development and in specific tissues in response to infection.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. A. Nucleotide sequence of cDNA for pleurocidin from winter flounder isolated from the skin library. The positions of primers used for PCR are underlined and the deduced amino acid sequence is shown in upper case letters below the nucleotide sequence. Arrows indicate the mature 5' and 3' termini of the pleurocidin peptide and diamonds indicate the positions of introns. The single *SstI* restriction endonuclease site (GAGCTC) and the putative polyadenylation site (aataaa) are indicated in boldface. *B.* Hydrophobicity plot of predicted pleurocidin polypeptide WF2 constructed using the Kyte-Doolittle option of DNA Strider (Marck 1992). The borders of the mature pleurocidin are indicated by vertical arrows. *C.* Diagrammatic representation of helical struc-

ture of predicted pleurocidin polypeptide WF2 constructed using the Helical Wheel routine of GCG. Hydrophobic residues and glycines are boxed and polar residues are not. The first amino acid (G) of the mature polypeptide is found at the top of the wheel.

Figure 2. Amplification of hepcidin cDNAs from halibut and salmon liver and spleen. RNA was prepared from tissues of fish infected with a bacterial pathogen to induce expression of antimicrobial peptide genes, reverse-transcribed and subjected to PCR using the primers listed below. Actin was run as a control to show expression of a house-keeping gene. The labelling on the figure is as follows: HL - halibut liver; SL - salmon liver; HS - halibut spleen; SS - salmon spleen; M – markers. For the primers 5'U is the Universal 5' primer used in all reactions, Sal is Hc Sal (below) and WF is HcPA3b (below).

HepUniversal 5': AAGATGAAGACATTCAGTGTTGCA

HcPA3 3'B2: GTTGTTGGAGCAGGAATCC

Hc Sal: TGCTGGCAGGTCCTCAGAATTTGC

Figure 3. Alignment of pleurocidin-like peptide sequences deduced from nucleotide sequences of genes and PCR products amplified from skin and/or intestine of the following species: winter flounder (WF), yellowtail flounder (YF), witch flounder (GC), American plaice (AP) and Atlantic halibut (AH).

Figure 4. PCR amplification of pleurocidin-like genes from winter flounder DNA. Amplification products (P) were resolved on a 1% agarose gel using the 100 bp ladder as molecular weight markers (M). Products visible as distinct bands are labeled WF1 (900 bp), WF2 (810 bp), WF3 (650 bp) and WF4 (510 bp).

Figure 5. Extended genomic sequence of WF4 obtained by PCR using primers PL1/PL2. introns are indicated in lower case and coding sequence in upper case. The positions of the primers PL1 and PL2 used for PCR are underlined.

Figure 6. Alignment of predicted polypeptide sequences of five winter flounder pleurocidin family members. Large vertical arrows indicate the positions where introns were found in the genomic sequences. The second intron of WF3, indicated by a small vertical arrow, is found more upstream than those of the other genes. The predicted polypeptide sequences of dermaseptin B1 (Amiche et al.

1994) and ceratotoxin B (Marchini et al. 1995) are shown below the pleurocidin family members. Boxed amino acids are shared by half of the sequences.

Figure 7. Expression of specific pleurocidin-like genes in different tissues of winter flounder. Tissues were esophagus (E), pyloric stomach (PS), cardiac stomach (CS), pyloric caeca (PC), liver (L), spleen (SP), intestine (I), rectum (R), gill (G), brain (B) and skin (SK). Markers (M) were the 100 bp ladder. Primers were specific to each pleurocidin variant (Table 2).

Figure 8. Reverse transcription-polymerase chain reaction assay of pleurocidin expression. Samples are from larvae (5 and 13 dph), metamorphosing larvae (20 dph), newly metamorphosed larvae (27 dph), juveniles (41 dph), skin from the lower (LS) and upper side (US) of the fish and tissue from the lower (LI) and upper (UI) intestine. Primers specific for pleurocidin (panel A) and actin (panel B) were used.

Figure 9. Expression of specific pleurocidin-like genes during winter flounder larval development. Samples are from larvae (5, 9 and 15 dph), metamorphosing larvae (20 dph), newly metamorphosed larvae (25, 30 and 36 dph) and juveniles (41 dph). Controls using the 5' or 3' primers alone and with no template (NT) are also shown. Primers were specific to each pleurocidin variant (Table 2).

Figure 10. Southern analysis of pleurocidin genes of winter flounder (WF), yellowtail flounder (YF), American plaice (AP) and Atlantic halibut (AH). Total genomic DNA (7.5 μg) was digested with *Bam*HI (B) or *Sst*I (S) and the fragments resolved on a 1.0% agarose gel. The blot was hybridized successively with probes corresponding to WF1, WF2, WF3, and WF4. Markers (M) are lambda DNA digested with *Sty*I (24.0, 7.7, 6.2, 3.4, 2.7, 1.9, 1.4, 0.9 Kb).

Figure 11. Schematic of genomic organization of pleurocidin-like genes and pseudogenes (ψ) from winter flounder. Introns are represented by solid boxes and exons by stippled boxes.

Figure 12. Locations of transcription factor binding sites upstream of pleurocidin genes and pseudogenes. Promoters are indicated by hatched boxes, introns by solid boxes and genes and exons by stippled boxes.

Figure 13. Survival of a Gram–positive bacterium (methicillin-resistant *Staphylococcus aureus* - MRSA) upon exposure to NRC-15 at its minimal inhibitory concentration (MIC) and ten times its MIC. *S. aureus* was grown in Mueller-Hinton broth and exposed to NRC-15 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

Figure 14. Survival of a Gram-negative bacterium (*Pseudomonas aeruginosa*) upon exposure to NRC-13 at its minimal inhibitory concentration (MIC) and ten times its MIC. *P. aeruginosa* was grown in Mueller-Hinton broth and exposed to NRC-13 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

Figure 15. Survival of a yeast (*Candida albicans*) upon exposure to NRC-12 at its minimal inhibitory concentration (MIC) and ten times its MIC. *C. albicans* was grown in Mueller-Hinton broth and exposed to NRC-12 at its MIC and ten times its MIC. At the specified intervals equal aliquots were removed from the culture, plated on MHB plates, and the resulting colonies were counted.

Figure 16. A. Nucleotide sequence of unspliced liver cDNA encoding Type I salmonid hepcidin. Exon sequences are indicated in upper case letters and the deduced amino acid sequence is shown below the nucleotide sequence. The gt/ag intron/exon boundaries are highlighted in boldface and the polyadenylation signal (aataaa) is underlined. B. Nucleotide sequence of partially spliced cDNA from halibut spleen encoding Type I salmonid hepcidin. C. Comparison of intron/exon structure in human, mouse and salmon. Exons are represented by hatched boxes and introns by a single line (sizes in bp shown beneath).

Figure 17. Alignment of winter flounder (WF1, WF2, WF3a, WF3b, WF4), Atlantic halibut (Hb1.1, Hb5.3, Hb7.5, Hb17, Hb357) and Atlantic salmon (Sal1, Sal2, Sal2.1, Sal8.6) hepcidins with those of Japanese flounder (JFL4, JFL6), medaka, hybrid striped bass and human. A partial sequence from rainbow trout (GenBank accession AF281354_1) is also shown. The predicted positions of signal peptidase and pre-protein cleavages are indicated by arrows.

Figure 18. Alignment of 3' untranslated regions of (A) winter flounder (WF1, WF2, WF3a, WF3b, WF4) and (B) Atlantic salmon (Sal1, Sal2) hepcidin cDNAs. Conserved nucleotides are boxed. The positions of the primers used to amplify hepcidin homologs from halibut and salmon are indicated by arrows.

Figure 19. Southern hybridization analysis of hepcidin in different fish species. *Sst*I digests of genomic DNA (7.5 μg) from hagfish (Hg), shark (Sh), white sturgeon (St), winter flounder (WF), yellowtail flounder (YF), American plaice (AP), witch flounder (Wi), Japanese flounder (JF), Atlantic salmon (AS), smelt (Sm) and haddock (Hd) were hybridized with Type I hepcidin from winter flounder. Size markers (M) are Lambda DNA digested with *StyI*.

Figure 20. Reverse transcription-PCR assay of hepcidin and actin gene expression in different tissues of winter flounder. Amplification products from adult winter flounder were amplified using gene-specific primers for Flatfish Type I (panel A), Type II (panel B) and Type III (panel C) hepcidins and for actin (310 bp) and resolved by electrophoresis on a 2% agarose gel. Markers (M) are the 100 bp ladder (BRL).

Figure 21. Reverse transcription-PCR assay of hepcidin and actin gene expression in different tissues of control Atlantic salmon (C) and those infected with *Aeromonas salmonicida* (I). Amplification products from reactions using gene-specific primers for Salmonid Type I (panel A) and Type II (panel B) hepcidins (163 bp) and for actin (400 bp) were resolved by electrophoresis on a 2% agarose gel. Markers (M) are the 100 bp ladder (BRL).

Figure 22. Reverse transcription-PCR assay of hepcidin and actin expression in developing winter flounder larvae. Samples were larvae at 5 dph (lane 1), 12 dph (lane 2), 19 dph (lane 3), 27 dph (lane 4), 41 dph (lane 5) and adult (lane 6). Amplification products from reactions using gene-specific primers for Flatfish Type I (panel A), Type II (panel B) and Type III (panel C) hepcidins and for actin (400 bp) were resolved by electrophoresis on a 2% agarose gel using a 100 bp ladder (Pharmacia) as markers (lane M).

DETAILED DESCRIPTION OF THE INVENTION

Fish Rearing

Winter flounder larvae were reared as described (Douglas, Gawlicka et al. 1999), the disclosure of which is incorporated herein by reference. Saint John River stock Atlantic salmon (*Salmo salar* L.) were maintained in single-pass, heated, dechlorinated fresh water at 12°C in the Dalhousie University Aquatron facility in Halifax, Nova Scotia. All fish were euthanised with an overdose of tricaine methanesulfonate (MS 222, 0.1 g L^{-1}, Argent Chemical Laboratories, Inc., Redmond, WA, USA) prior to sampling. All animal procedures were approved by the Dalhousie University Committee for Laboratory Animals and the National Research Council - Halifax Local Animal Care Committee.

Bacterial Challenge

Aeromonas salmonicida subsp *salmonicida* strain A449 (Trust et al. 1983) was cultured to mid-logarithmic growth in Tryptic Soy Broth (TSB) at 17°C. The absorbance at 600nm of the bacterial suspension was determined and the bacteria were resuspended to approximately 5 x 10^7 cfu mL^{-1} in sterile Hanks Balanced Salt Solution (HBSS). Three salmon (200g each) were anaesthetised with 50 mg L^{-1} TMS, injected intraperitoneally with 2.5 x 10^6 cfu bacteria in 50 µL HBSS and allowed to recover in fresh water. Uninjected fish from the same cohort were maintained in separate tanks as controls. Three days post-injection, control and infected salmon were euthanised as described above and samples of tissues removed. Blood was drawn from the caudal vein into a heparinised container. To confirm that the fish were positive for *A. salmonicida*, the posterior kidney of both infected and control fish were swabbed and used to inoculate tryptic soy agar (TSA) that was incubated at room temperature overnight. Atlantic halibut tissue samples were obtained from a bacterial challenge study performed at Bedford Institute of Oceanography, Dartmouth, Nova Scotia.

Sampling

Tissues (oesophagus, stomach, pyloric caecae, liver, spleen, intestine, anterior kidney, posterior kidney, gill, skin, ovary, rectum, heart, muscle and brain) were removed into RNALater (Ambion, Austin, TX, USA) and kept at -80° C until used. Samples of winter flounder larvae at different stages and juveniles were rinsed in

RNALater (Ambion, Austin, TX, USA), transferred into 1.5 ml Eppendorf tubes containing 0.5-1.25 ml RNALater, and kept at -80° C until used.

Pleurocidins

Isolation of pleurocidin cDNA

A cDNA library constructed from winter flounder skin (Gong et al 1996) was screened using degenerate oligonucleotides (PleuroA, PleuroB; Table 1). The library was plated at 80,000 phage/plate and duplicate lifts to HyBond filters were made of each of eight plates. A mixture of radioactively end-labelled PleuroA and PleuroB probes was hybridised with the filters at 50° C using standard procedures, and the filters were washed in 1X SSC/0.1% SDS at 50° C for 45 min. Plaques that showed matching hybridization signals on both duplicate filters were picked and the library rescreened until 100% purity of the recombinant plaques was obtained. Two recombinants were completely sequenced using an ABI373 stretch automated sequencer and the AmpliTaqFS Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Foster City, CA, USA). Sequence data were analyzed using Sequencher (Gene Codes, Inc., Ann Arbor, MI, USA) and DNA Strider. The amino-terminal signal sequence was predicted using SignalP (http://www.cbs.dtu.dk/services/SignalP). The Helical Wheel routine of the GCG package (http://www.gcg.com) was used to model the helical structure of the predicted antimicrobial peptide sequences.

Genomic PCR

Genomic sequences were amplified using two sets of primers specific to the winter flounder pleurocidin cDNA (PL1/PL2 and PL5'/PL3'; Table 1; Fig. 1). The amplification conditions were: 1 min at 94° C; 35 cycles of 30 s at 94° C; 30 s at 52° C, 90 s at 72° C; and 2 min at 72° C, and products were resolved on a 1% agarose gel. Bands were excised from the gel, extracted using Gene-Clean (Bio101, La Jolla, CA, USA) and cloned into the Topo TA2.1 vector (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturers. Several isolates from each transformation were sequenced and analyzed as described above. Intron positions were identified by comparison with the cDNA sequence.

Identification of additional winter flounder pleurocidin-like transcripts by RT-PCR

Total RNA was isolated from winter flounder skin and intestine as described. Reverse transcription of 2 μg of total RNA was performed using the RETROScript kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendation. PCR was performed using PL3' and a primer corresponding to the amino terminus of the precursor polypeptide (PL5'; Table 1). The amplification conditions were: 1 min at 94° C; 32 cycles of 30 s at 94° C; 30 s at 50° C, 90 s at 72° C; and 2 min at 72° C and products were resolved on a 2% NuSeive gel. Bands were excised, cloned and sequenced as described above.

Tissue-specific gene expression

Tissue-specific expression of pleurocidin was investigated by northern analysis using polyadenylated RNA (500 ng) from adult skin, liver, ovary, muscle, spleen, pyloric caeca, stomach and intestine. The entire insert from the cDNA clone corresponding to WF2 was radioactively labelled and incubated with the blot overnight at 60° C in UltraHyb hybridisation solution (Ambion, Austin, TX, USA). The blot was washed to a stringency of 50° C in 1X SSC/0.1% SDS for 1 h before exposure to X-ray film. RT-PCR was also employed using primers specific to WF1, WF1a, WF2, WF3, WF4, WFYT and WFX (Table 2) to assay expression of the different pleurocidin-like variants in various tissues. The conditions used were as described in the preceding paragraph except that the annealing temperature was 52 ° C.

Developmental expression

Two larval time series were used to assess developmental expression of pleurocidin-like genes. In the first, RNA was isolated from pooled samples of twenty whole larvae (5 and 13 dph), ten whole metamorphosing larvae (20 dph) and newly metamorphosed larvae (27 dph), gut tissue of two juveniles (41 dph), skin from the upper and lower side of adult fish and tissue from adult upper and lower intestine. RNA was isolated as described (Douglas, Gawlicka et al. 1999), the disclosure of which is incorporated herein by reference, and the assays were performed using the primers PL5' and PL2 and conditions described above for RT-PCR. Amplification of the actin mRNA was performed as previously described (Douglas, Bullerwell et al. 1999), the disclosure of which is incorporated herein by reference, to confirm the

level of expression of a housekeeping gene and to provide an internal control for pleurocidin expression. In the second larval time series, RNA was isolated from pooled samples of twenty whole larvae (hatch, 5 and 9 dph), ten whole larvae (15, 20, 25, 30 and 36 dph) and gut tissue of two juveniles (41 dph). Assays were performed using primers specific to WF1, WF1a, WF2, WF3, WF4, WFYT and WFX (Table 2) to determine expression of the different pleurocidin-like variants at different stages of development. The conditions used were as described in the preceding paragraph.

Southern analysis

Southern analysis of *Bam*HI- and *Sst*I-digested genomic DNA from winter flounder, three other flatfish (American plaice *Hippoglossoides platessoides* Fabricius, Atlantic halibut *Hippoglossus hippoglossus* L. and yellowtail flounder *Pleuronectes ferruginea* Storer), haddock (*Melanogrammus aeglefinus* L.), pollock (*Pollachius virens* L.) and smelt (*Osmerus mordax* Mitchill) was performed sequentially using the entire inserts from genomic clones corresponding to WF1, WF2, WF3 and WF4 as probes. Hybridisations were performed overnight at 65° C as previously described (Douglas, Gallant et al. 1998), the disclosure of which is incorporated herein by reference, and the blots were washed at 65° C in 0.5X SSC/0.1% SDS for 1 h and exposed to X-ray film. Blots were stripped by incubating twice in boiling 0.5% SDS and checked for residual signal by exposure to X-ray film overnight.

Amplification of pleurocidin-like sequences from other fish species

Total RNA was isolated from skin and intestine of yellowtail flounder, witch flounder and Atlantic halibut and reverse-transcribed as described above (**RT-PCR analysis**). Total genomic DNA was isolated from milt of yellowtail flounder, witch flounder, American plaice and Atlantic halibut. Two sets of primers specific to the winter flounder pleurocidin cDNA (PL1/PL2 and PL5' /PL3'; Table 1; Fig. 1) were used and the amplification conditions were: 1 min at 94° C; 32 cycles of 30 s at 94° C; 30 s at 50° C, 90 s at 72° C; and 2 min at 72° C. Products were resolved on a 2% NuSeive gel, bands excised, cloned and sequenced as described above.

Identification of pleurocidin-like sequences in the winter flounder genome

A winter flounder genomic λ-GEM library was screened using a radioactively labeled probe for pleurocidin (WF2; Douglas et al., 2001). Four clones were picked and replated until 100% purity was achieved. The clones were mapped using *BamHI, SstI, XhoI* and *Eco RI* and two clones (λ1.1 and λ5.1) that differed in restriction pattern were selected for sequencing. Both clones were completely sequenced using an ABI373 stretch automated sequencer and the AmpliTaqFS Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer, Foster City, CA, USA. Transcription factor binding sites were identified using WWW Signal Scan (http://bimas.dcrt.nih.gov/molbio/signal/) with the TransFac and TFD databases and promoters were detected using the eukaryotic promoter prediction by neural network software available at the Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html).

Hepcidins

Molecular Characterisation of Hepcidin cDNAs

Eight ESTs showing high similarity to human hepcidin were identified from the winter flounder EST database (Douglas, Gallant et al. 1999) and four from the Atlantic salmon database (Douglas, Tsoi et al. 2002). Using these sequences to screen dbEST, BLASTX analysis revealed two related sequences from Japanese flounder (C23298.1 and C23432.1), one sequence from rainbow trout (AF281354_1) and five identical sequences from medaka (AU178966, AU179222, AU179314, AU179768 and AU180044). Sequence data were analyzed using Sequencher (Gene Codes, Inc., Ann Arbor, MI, USA) and DNA Strider (Marck 1992). Alignments and similarity matrices were calculated using ClustalW (Thompson, Higgins et al. 1994) and graphically visualised using SeqVu (Garvan 1996). The on-line servers PSORT (http://PSORT.nibb.ac.jp), Compute pI (http://expasy.hcuge.ch/cgi-bin/pi_tool), and Network Protein Sequence @nalysis (http://npsa-pbil.ibcp.fr/cgi-bin/secpred_consensus.pl) were used to predict N-terminal signal sequences, pI and secondary structure, respectively. The secondary structure prediction program utilized seven different algorithms (for details, see web site) and provided a consensus prediction based on these results.

Southern Hybridisation

Total genomic DNA was prepared from winter flounder (*Pleuronectes americanus*), yellowtail flounder (*Pleuronectes ferruginea*), witch flounder (*Glyptocephalus cynoglossus*), Japanese flounder (*Paralichthys olivaceus*), American plaice (*Hippoglossoides platessoides*), Atlantic salmon (*Salmo salar*), haddock (*Melanogrammus aeglefinus*), smelt (*Osmerus mordax*), hagfish (*Eptatretus burgeri*), tiger shark (*Scyliorhinus torazame*) and white sturgeon (*Acipenser transmontanus*) as previously described (Douglas, Bullerwell et al. 1999), the disclosure of which is incorporated herein by reference. DNA (7.5 µg) was digested with *SstI* according to the manufacturer's recommendations and the fragments resolved on a 1% agarose gel. A 104 bp probe corresponding to amino acid residues WMENPT. . . .GCGFCC of Type I winter flounder hepcidin was labeled using the DIG Labelling Kit (Roche Applied Science, Laval, PQ, Canada) and hybridized to the membrane for 2h at 42 °C using the Easy Hyb kit (Roche Applied Science, Laval, PQ, Canada). The membrane was washed in 0.2X SSC at 65 °C and signal detected using the DIG Luminescent Detection Kit (Roche Applied Science, Laval, PQ, Canada).

Expression of Hepcidin Genes by RT-PCR

Primers were designed based on the cDNA sequences determined in this study (Table 3). Amplification of actin mRNA was performed to confirm the steady-state level of expression of a housekeeping gene and provide an internal control for the hepcidin gene expression analyses. Controls were performed using single primers to eliminate single primer artifacts and without reverse transcription to eliminate amplification products arising from contaminating genomic DNA.

Total RNA was isolated from tissues of uninfected adult winter flounder and uninfected and infected adult salmon and halibut using the RNAWiz Kit (Ambion, Austin, TX, USA) according to the manufacturer's recommendations. Tissues were homogenized using a 7mm generator on a Polytron standard rotor stator homogenizer (Kinematica). In addition, RNA was isolated from pooled samples of twenty whole larvae (hatch, 5 and 9 dph), ten whole larvae (15, 20, 25, 30 and 36 dph), gut tissue of two juveniles (41 dph) and adult winter flounder liver. To eliminate contaminating DNA, the Ambion DNA-*free* TM protocol was used as directed. Briefly, 4 units of DNase 1 was added to the resuspended RNA and incubated for 1 hour at 37C. After

incubation, DNAse Inactivation Reagent was added to remove the enzyme and RNA concentrations were determined using a Beckman DU-64 Spectrophotometer.

First strand cDNA was synthesized from 1 μg of total RNA using the Retro-Script kit (Ambion, Austin, TX, USA) and aliquots of the reaction products were subjected to PCR using rTaq polymerase (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or the Advantage2 PCR kit (Clontech, Palo Alto, CA, USA). The primers and annealing temperatures are listed in Table 3. The amplification conditions were: 1 min at 95° C; 32 cycles of 15 s at 95° C; 30 s at the annealing temperature, 30 s at 68° C; hold at 4° C. Amplification products were resolved on a 2% NuSieve agarose gel with a 100 bp ladder as a marker (Gibco BRL, Gaithersburg, MD, USA) and the amount of each product was quantified using a GelDoc 1000 video gel documentation system (BioRad, Mississauga, Ont., Canada) with the Multianalyst software.

Amplification of hepcidin-like sequences from other fish species

Total RNA was isolated from liver and spleen Atlantic halibut and Atlantic salmon and reverse-transcribed as described above (**RT-PCR analysis**). Two sets of primers were used (see legend, Fig. 2) and the amplification conditions were: 2 min at 94° C; 32 cycles of 30 s at 94° C; 30 s at 52° C, 30 s at 72° C; and 2 min at 72° C. Products were resolved on a 2% NuSeive gel, bands excised, cloned and sequenced as described above.

Prediction and assessments of antimicrobially active peptide sequences

Prediction of active cationic peptide sequences

The mature peptide sequences from Figure 3 (pleurocidin-like peptide sequences deduced from nucleotide sequences of genes and PCR products amplified from fish tissues) constituted the basis of sequence selection. Generally, upon extensive sequence analysis, those peptides that possessed a net positive charge and had their hydrophilic and hydrophpobic residues well-separated in models were produced. Also, generally those peptide genes that were likely to be expressed (possessed promoters) were used, although pseudogene controls were also included in the panel. The exact start/end residues were decided upon based on several factors listed below. In most cases the N-terminus of the mature peptide was well defined, since it followed

directly the conserved signal peptide region, and aligned well with other mature peptides. Wherever a straightforward determination on the N-teminal amino acid was not possible, an attempt was made to preserve GW or GF at the N-terminus, as this is frequently encountered among cationic peptides. In addition, two versions of WF1a (NRC-2 and NRC-3) were produced: one contained N-terminal GRRKRK, and the other did not. In some cases the C-terminus of the mature peptide was also well defined, since it was followed directly by a conserved acidic propiece. However significant ambiguity as to the C-terminal amino acid existed among many peptides. Generally, two rules were followed in deciding upon C-terminal amino acids: (1) wherever glycine appeared at or near the C-terminus, it was considered to be a precursor for carboxy–terminus amidation; (2) large numbers of negatively charged amino acids near the C-terminus were generally considered to be a part of the propiece and not the mature active peptide, and were not included in the sequence.

All antimicrobial peptides used in this study were synthesized by N-(9-fluorenyl) methoxy carbonyl (Fmoc) chemistry at the Nucleic Acid Protein Service (NAPS) unit at the University of British Columbia. Peptide sequences are shown in Table 4. Peptide purity was confirmed by HPLC and mass spectrometry analysis in each case. In the case of NRC-7 further purification by RP-HPLC was performed until homogeneity of the sample was obtained.

Bacterial Strains and *Candida albicans*

All strains used in this study are listed in Table 4a. Most non-fish bacterial strains as well as *Candida albicans* were grown at 37°C in Mueller-Hinton Broth (MHB; Difco Laboratories, Detroit), while the fish bacteria were maintained at 16°C in Tryptic Soy Broth (TSB; Difco, 5g/l NaCl). All strains were stored at –70°C until they were thawed for use and sub-cultured daily. The following strains, *P.aeruginosa* K799 (parent of Z61), *P.aeruginosa* Z61 (antibiotic supersusceptible), *Salmonella typhimurium* 14028s (parent of MS7953s), *Salmonella typhimurium* MS7953s (defensin supersusceptible), as well as *Staphylococcus epidermidis* (human clinical isolates) and methicillin-resistant *Staphylococcus aureus* (MRSA; isolated by Dr. A. Chow, University of British Columbia) have been kindly donated by Prof R.E.W. Hancock, University of British Columbia.

Escherichia coli strain CGSC 4908 (*his-67, thyA43, pyr-37*), auxotrophic for thymidine, uridine, and L-histidine (Cohen *et al.*, 1963) was kindly supplied, free of charge, by the *E.coli* Genetic Stock Centre (Yale University, New Haven, CT). MHB supplemented with 5 mg/L thymidine, 10 mg/L uridine and 20 mg/L L-histidine (Sigma Chemical Co., St. Louis, MO), was used to grow *E.coli* CGSC 4908 unless otherwise specified.

Two field isolates of the salmonid pathogen *Aeromonas salmonicida* are from the IMB strain collection.

Minimum Inhibitory Concentrations

The activities of the antimicrobial peptides were determined as minimal inhibitory concentrations (MICs) using the microtitre broth dilution method of Amsterdam (Amsterdam, 1996), as modified by Wu and Hancock (1999). Serial dilutions of the peptide were made in water in 96-well polypropylene (Costar, Corning Incorporated, Corning, New York) microtiter plates. Bacteria or *C. albicans* were grown overnight to mid-logarithmic phase as described above, and diluted to give a final inoculum size of 10^6 cfu/ml. A suspension of bacteria or yeast was added to each well of a 96 well plate and incubated overnight at the appropriate temperature. In the case of *E. coli* CGSC 4908, supplemented MHB was used. Inhibition was defined as growth lesser or equal to one-half of the growth observed in control wells, where no peptide was added. Three repeats of each MIC determination were performed.

Killing assays

Survival of bacteria and *C. albicans* upon exposure to selected peptides applied at their minimal inhibitory concentrations (MICs) and ten times their MICs was measured using standard methodology. The test organisms were grown in MHB and exposed to the peptides. At the specified time intervals equal aliquots were removed from the cultures, plated on MHB plates, and the resulting colonies were counted. Percentage survival was plotted against time on a logarithmic scale. Two repeats of each experiment were performed.

RESULTS

Pleurocidins

cDNA sequence

The two clones isolated from the skin cDNA library were identical in sequence to each other and to the genomic PCR product WF2 (see below). They contain 356 bp and encode an open reading frame of 68 amino acids (Fig. 1A). There is a 5'-untranslated region of 26 bp and a 3'-untranslated region of 84 bp, excluding the polyA tail. A canonical polyadenylation signal AATAAA is found 22 bp upstream of the polyA tail. The first 22 amino acids of the open reading frame form a highly hydrophobic domain (Fig. 1B) predicted to be a signal peptide with a cleavage site that precisely matches the amino terminus of the mature pleurocidin. The predicted amino acid sequence of residues 23-47 exactly matches the published amino acid sequence of mature pleurocidin (arrows, Fig. 1A). The mature peptide can assume an amphipathic helix that contains a predominance of positively charged amino acids on one face and hydrophobic amino acids on the other (Fig. 1C). The carboxy-terminal 21 amino acids form a negatively charged domain that is not present in the mature pleurocidin, confirming the recent report of Cole et al. (2000).

Genomic PCR

Four distinct bands (WF1-4) were amplified using primers PL5' and PL3' (Fig. 4). Sequence analysis of each product was consistent with the sizes of the bands and verified that each amplification product was different (Table 5). Two distinct bands were amplified using primers PL1 and PL2 that corresponded to WF2 and WF4 containing additional upstream and downstream sequence (data not shown). When the intron sequences were removed, the sequence of WF2 exactly matched that of the pleurocidin cDNA clone isolated from the skin library (Fig. 1A).

All four of the pleurocidin-like genes contained two introns within the coding sequence and three of the genes showed identical intron locations (WF1, WF2 and WF4). However, the position of the second intron in WF3 occurred upstream of those of the other genes, resulting in a shorter second exon and longer third exon. The sizes and sequences of the introns varied among the four pleurocidin genes (Table 5). Evi-

dence from the two more extensive genomic sequences of WF2 and WF4 obtained using primers PL1 and PL2 indicates that a third intron immediately upstream of the initiation codon is also a feature of this gene family (Fig. 5). This was also noted for the genomic sequence reported by Cole et al (Cole, Darouiche et al. 2000).

An alignment of the predicted amino acid sequences is shown in Fig. 6. The positions of the introns (indicated by vertical arrows) were determined by comparison with the corresponding RT-PCR and cDNA-derived sequences. The positions of the mature peptide were determined by comparison with the published amino acid sequence of pleurocidin (Cole, Weis et al. 1997). All of the predicted mature polypeptides could assume amphipathic α-helical structures similar to that shown in Fig. 1C, although the positively charged portions were not as striking in WF1 and WF3 as in WF2 and WF4 (data not shown).

Tissue-specific expression

Northern analysis was only able to detect pleurocidin transcripts in skin (data not shown). However, the more sensitive RT-PCR assay indicated that pleurocidin was also expressed in other tissues, particularly gill and gut. Using primers PL5' and PL3', two bands were obtained from winter flounder skin (265 and 175 bp) and two from intestine (215 and 175 bp). Sequence analysis of several clones of each size showed that the 265 bp winter flounder skin clones corresponded to the genomic sequence of WF1 when intron sequences were removed (Table 6). Five of the 175 bp clones from skin and two of the 175 bp clones from intestine corresponded to the genomic sequence of WF2. This is consistent with results of northern analysis using the cDNA clone corresponding to the WF2 probe that showed hybridisation only to 200-nucleotide mRNA from the skin (data not shown). On the other hand, nine of the 175 bp clones from intestine and four of the 175 bp clones from skin corresponded to the genomic sequence of WF3. No RT-PCR products were obtained that corresponded to WF4. All seven of the 215 bp intestine clones corresponded to a novel family member (WF1a) not represented by any of the winter flounder genomic sequences determined in this study.

Using primers specific to each of the pleurocidin-like variants reported above, as well as to additional pleurocidin-like variants identified on Lambda clones, we were able to demonstrate that different variants were expressed in different tissues (Fig. 7). WF2, WF3 and WFYT showed the expression in the widest distribution of

tissues, whereas WF1 and WF4 were expressed in mainly in the gill and skin, and WFX was only expressed in the skin. Transcripts of WF1a could not be detected in any tissue.

Developmental expression

Using primers PL5' and PL2 (Table 1) from highly conserved regions of the pleurocidin-like peptides, low levels of transcripts were evident at 5 dph and increased during development (Fig. 8). Strong signals were obtained from adult skin and weak signals from intestinal tissue. Expression of the housekeeping gene, actin, was relatively constant throughout development.

Using primers specific to each of the pleurocidin-like variants reported above, as well as to additional pleurocidin-like variants identified on Lambda clones, we were able to demonstrate that different variants were expressed at different times during development (Fig. 9). WFX transcripts were only detectable at 20 dph, and WF2, WF3 and WFYT were detectable in premetamorphic larvae and metamorphic juveniles. No expression of WF1 and WF4 was detectable at any stage of development.

Southern analysis

Positive signals were specific to flatfish DNA using the WF1, WF2, WF3 and WF4 genomic probes (Fig. 10). No signals were detected with haddock, pollock or smelt DNA (data not shown). All four probes showed hybridisation to common *Sst*I and *BamHI* bands from the DNAs of all four flatfish, indicating that the genes are clustered on these genomes. The sizes of the hybridising fragments from the winter flounder digest are given in Table 7.

Amplification of pleurocidin-like sequences from other fish species

An alignment of the deduced amino acid sequences of pleurocidin-like peptides from American plaice, yellowtail flounder, witch flounder and Atlantic halibut is shown in Fig. 3. High conservation is present in the signal peptide and acidic propiece regions, whereas the portion corresponding to the mature peptide shows much more variability.

Identification of pleurocidin-like sequences in the winter flounder genome

Two clones containing fragments of 12.5 and 17.5 kb, respectively, were isolated from a genomic library from winter flounder. The 12.5 kb fragment encoded the gene corresponding to WF2 and two pseudogenes. The 17.5 kb fragment encoded the gene corresponding to WF1, one pseudogene and two previously undescribed pleurocidin-like sequences referred to as WFX and WFYT. A schematic of the gene arrangement is shown in Fig. 11. Scanning of the sequences upstream of the coding sequence revealed a canonical eukaryotic promoter, TATA and CAAT boxes as well as highly conserved sites for several transcriptions factors including NF-IL6, AP1 and α-interferon (Fig. 12). No promoter sequences were identified upstream of pseuodgenes.

Prediction and assessment of antimicrobially active peptide sequences

The minimal inhibitory concentrations of the chemically produced peptides against a wide range of baterial pathogens and *C. albicans* were determined and are shown in Table 8. Generally speaking many peptides showed the ability to inhibit the growth of a broad spectrum of bacterial pathogens and *C. albicans*. Particularly good examples of peptides with a broad spectrum of antimicrobial activity are the three peptides derived from American plaice (NRC-11, NRC-12, and NRC-13) and three peptides derived from witch flounder (NRC-15, NRC-16, and NRC-17). Of those, NRC-15, NRC-13, and NRC-12 showed ability to kill methicillin-resistant *S. aureus* (Fig. 13), *P. aeruginosa* (Fig. 14) and *C. albicans* (Fig. 15), respectively.

In addition to demonstrating that pleurocidin-like peptides are active against a wide range of bacteria as well as *C. albicans*, our results indicate which factors should be considered in selecting antimicrobially active peptides from genomic sequences.

Firstly, a notable group of peptides with poor or no activities in our hands were peptides derived from pseudogenes (NRC-8, NRC-9, NRC-10). These results indicate that peptides capable of being expressed in the host organism may be better candidates for antimicrobials.

Secondly, the previously described N-terminal GRRKRK in WF1a (Fig. 2) proved to be the determinant of antimicrobial activity in NRC–3 as shown by the fact NRC-2 (identical to NRC-3 but missing the aforementioned fragment) was only marginally active (Table 8). This result stresses the importance of carefully selecting the start/end residues in the mature peptide, wherever these are not apparent in the

start/end residues in the mature peptide, wherever these are not apparent in the original pre-pro-sequence.

Lastly, the previously described principles of: selecting positively charged peptides with good separation of hydrophilic and hydrophobic residues in helical wheel models, preserving GW or GF at the N-terminus, amidating the C-terminus where glycine was present, and cropping off clusters of acidic C-terminal amino acids appeared to be successful in selecting antimicrobially active peptides.

Hepcidins

Bacterial Challenge

Three days post-injection, the infected Atlantic salmon were lethargic and anorexic. On sampling, the posterior kidneys of the injected fish were positive for *A. salmonicida* whereas those of the control fish were not.

Molecular Characterisation of Hepcidin cDNAs

Although the winter flounder EST database contains sequences from liver, ovary, stomach, intestine, spleen and pyloric caecae cDNA libraries and the Atlantic salmon EST database contains sequences from liver, head kidney and spleen, hepcidin-like sequences were only detected in spleen and liver cDNA libraries of both fish. Four of 135 ESTs (3.0%) in the winter flounder liver library and two of 281 ESTs (0.7%) in the winter flounder spleen library encoded hepcidins. Three of 982 (0.3%) ESTs in the Atlantic salmon liver library encoded hepcidins. Five hepcidin sequences were also found in subtracted spleen (1.8%) and three in subtracted liver (0.6%) Atlantic salmon cDNA libraries that were enriched in transcripts up-regulated during infection with *Aeromonas salmonicida* (Douglas et al., unpub). Unfortunately, since these are subtracted libraries, the inserts are only portions of the complete transcripts.

Analysis of the nucleotide sequences of Atlantic salmon hepcidin cDNAs revealed that one salmon EST (SL1-0412) was approximately 300 nucleotides longer than the other two. Furthermore, the hepcidin coding sequence was incomplete. Complete sequencing of this clone revealed the presence of two introns with standard

GT/AG splice junctions (Fig. 16A). When removed, an open reading frame encoding a complete hepcidin-like peptide was obtained. Similarly, an incompletely spliced halibut transcript was amplified that still retained the second intron (Fig. 16B). Compared to mammals, the introns of salmon and probably halibut are in similar locations but of shorter length (Fig. 16C). In addition to these incompletely spliced cDNAs, we identified a winter flounder EST (WF4) that contains a large deletion relative to the other sequences that corresponded closely to the second exon of salmon and human hepcidin. Assuming the intron positions are conserved among vertebrates, this deletion could correspond to the removal of exon2, and resulted in a peptide that differed from WF3a and WF3b in only five amino acid postions of the remaining peptide.

The deduced amino acid sequences of five different winter flounder hepcidin cDNAs and two different Atlantic salmon hepcidins were aligned with those extracted from dbEST corresponding to Japanese flounder (two), medaka (one) and rainbow trout (one), as well as the recently reported hepcidin from hybrid striped bass (Shike et al. 2002). The sequences obtained from spleen and liver of Atlantic salmon (Sal2.1 and Sal8.6) and Atlantic halibut (Hb1.1, Hb5.3 and Hb7.5) by PCR are also included (Fig. 17). Human hepcidin was included as a representative of the mammals. The position of cleavage by signal peptidase was predicted by PSORT and the RX(K/)R motif typical of propeptide convertases (Nakayama 1997) was identified (vertical arrows; Fig. 17). The signal peptide sequence is 22-24 amino acids and is highly conserved among all of the fish sequences. The anionic propiece is 38-40 amino acids, depending on the particular hepcidin variant. The processed hepcidins contain 19-27 amino acids and all are positively charged at neutral pH except WF2 (Table 9). Types I and III hepcidin from flatfish as well as salmon type hepcidin contain eight cysteine residues in the mature peptide, which have been proposed to form four disulphide bonds. Type II winter flounder hepcidin is missing two cysteine residues, indicating that a maximum of three disulphide bonds could form. Hb357 contains only five cysteine residues and is quite different from the remaining hepcidin-like sequences. Results of secondary structure prediction methods indicated that the consensus structure of fish hepcidins was mostly random coil, although short stretches of extended strand were predicted by some methods.

Based on the alignment, it is apparent that there are at least three different groups of flatfish hepcidins distinguishable by shared insertions and deletions. WF2 and JFL6 (Flatfish Type II) share a deletion of seven amino acids near the KR cleav-

age site resulting in a processed peptide of 19 amino acids, whereas WF3a, WF3b, WF4, Hb1.1, Hb17, Hb5.3 and Sal8.6 (Flatfish Type III) exhibit a deletion of only four amino acids (excluding the portion corresponding to the missing exon of WF4) resulting in processed peptides of 22 amino acids. WF1 and JFL4 (Flatfish Type I) do not contain this deletion but do contain an insertion relative to all other reported hepcidins at a position adjacent to the signal peptidase cleavage site. In addition, WF1, bass and medaka share an insertion of one amino acid within the mature peptide relative to all other reported hepcidins, giving a peptide of 26-27 amino acids. WF3a and WF3b differ from each other by only one amino acid although they contain several silent substitutions and differences in the 5' and 3' untranslated regions. Hb357 represents a possible fourth class of flatfish hepcidins. The 3' untranslated regions of WF2 and WF1 are very different from those of the other hepcidin transcripts, WF2 containing a long additional portion relative to the others and WF1being shorter and less highly conserved (Fig. 18A).

The salmonid hepcidin-like peptides fall into one group; the four reported sequences all share two deletions and differ from each other by four amino acids in the mature peptide and four amino acids in the upstream pre-protein portion. The 3' untranslated regions of the salmon hepcidins are only moderately conserved (Fig. 18B).

Genomic Organisation of Winter Flounder Hepcidin Genes

Southern hybridization analysis of genomic DNA from a wide variety of fish with a probe corresponding to Type I hepcidin identified bands in all flatfish tested but none of the other fish species (Fig. 19). In winter flounder, two fragments of 4.3 and 4.5 kb hybridized with the probe. Two fragments of yellowtail flounder of identical size hybridized (4.3 kb) and two fragments of witch flounder genomic DNA also hybridized (4.3 and 20 kb), whereas only one fragment (4.3 kb) of the other flatfish genomic DNA hybridized.

Expression of Hepcidin Genes by RT-PCR

TISSUE-SPECIFIC GENE EXPRESSION

The results of RT-PCR assays of tissue-specific expression of the three winter flounder hepcidins are shown in Fig. 20. Type I hepcidin was abundantly expressed in the liver and, to a lesser extent, in the cardiac stomach. Type II hepcidin could not be

detected in any tissues, whereas Type III hepcidin was moderately expressed in the esophagus, cardiac stomach, and liver.

In uninfected Atlantic salmon, Type I hepcidin was expressed at quite high levels in the liver, blood and muscle, at low levels in gill and skin, and at barely detectable levels in anterior and posterior kidney (Fig. 21A, Table 10). Type II hepcidin was expressed at barely detectable levels in the gill and skin only (Fig. 21B). However, fish infected with *Aeromonas salmonicida* showed expression in most tissues tested (see below).

PATHOGEN-INDUCED GENE EXPRESSION

The expression of Type I hepcidin was up-regulated (↑or ratio of infected:control was greater than 2 in Table 10) in the esophagus, stomach, pyloric caecae, liver, spleen, intestine, posterior kidney, rectum and muscle. Weak up-regulation (ratio of infected: control was between 1 and 2) was also found in the anterior kidney and skin. The expression of Type II hepcidin was highly up-regulated (↑↑ or↑↑↑ in Table 10) in stomach, pyloricaecae, liver, spleen, intestine, brain, heart and muscle and weakly up-regulated (↑; ratio of infected:control was between 1 and 2) in esophagus, anterior and posterior kidney, skin and rectum. Neither hepcidin appeared to be expressed in gonad of either control or infected fish.

DEVELOPMENTALLY PROGRAMMED GENE EXPRESSION

RT-PCR analysis of hepcidin gene expression in winter flounder larvae of different ages is shown in Fig. 22. Transcripts of Type II hepcidins could not be detected at any stage of development, whereas Type I and Type III hepcidins were detectable in pre-metamorphic larvae. Type I hepcidin was more abundantly expressed than Type II hepcidin and was also expressed at an earlier time (5 dph vs. 9 dph.).

Amplification of hepcidin-like sequences from other fish species

Using a primer based on highly conserved sequences in the signal peptide of all reported hepcidins (Hep Universal 5') in combination with primers based on highly conserved sequences in the 3' UTR of salmon (HcSal 3') and flatfish (HcPA3b 3'), it was possible to amplify hepcidin-like sequences from the liver and spleen of halibut and salmon (Fig. 2). An alignment of the deduced amino acid sequences of

hepcidin-like peptides from winter flounder, Atlantic halibut and Atlantic salmon is shown in Fig. 17. Interestingly, flatfish-type hepcidin could be amplified from salmon (S8.6) and salmon-type hepcidin could also be amplified from a flatfish (Hb7.5).

DISCUSSION

Pleurocidins

Most antimicrobial peptides, including cecropins and dermaseptins, are encoded by multigene families that have probably arisen by sequential gene duplications. We have demonstrated that the winter flounder, and probably other flatfish, possess a gene family encoding antimicrobial compounds similar to pleurocidin. Comparison of the genomic amplification products obtained using PL1/2 with the cDNA sequence (Fig. 1A) showed that WF2 and WF4 contain three introns, the first of which occurs only 1 bp upstream from the initiator methionine. The second and third introns both occur within the mature peptide. The genes for GLa, xenopsin, levitide and caerulein – all skin peptides from *Xenopus laevis* – also contain an intron 1 bp upstream from the initiator methionine (Kuchler et al 1989). The intron positions are conserved in all but WF3 (Fig. 6), but they differ dramatically in size (Table 5), indicating that a considerable period of evolutionary time has elapsed since the duplication events occurred, or that the intron sequences are relatively free to drift.

Southern analysis shows that WF1-4 probes hybridise to other flatfish, including yellowtail flounder, Atlantic halibut and American plaice, but not to haddock, smelt or pollock. This hybridisation could be due to the highly conserved signal sequence and anionic portion which we have shown to be conserved in sequences isolated from these flatfish. Flatfish may provide a rich reservoir of potential therapeutants for the aquaculture industry. The probes for the different pleurocidin family members often recognise the same restriction fragments in winter flounder DNA, indicating that they may be clustered at a single locus on the genome. Complete sequencing of two Lambda clones hybridizing to pleurocidin confirms that such clustering does in fact occur (Fig. 11). Clustering of antimicrobial peptide genes has also been noted for insect cecropins (Gudmundson et al. 1991) and apidaecins (Casteels-Jossen et al. 1993), among others.

All of the members of the pleurocidin family are encoded as prepropolypeptides consisting of an amino-terminal signal sequence followed by the active peptide

and ending with an acidic portion. The deduced amino acid sequences of the signal and acidic sequences are very highly conserved whereas those of the predicted mature antimicrobial peptides are more variable (Fig. 6). All, however, appear to fold into amphipathic α-helices. This sequence conservation has allowed us to use a genomic approach to identify many different members of the pleurocidin gene family, not only from winter flounder but also from a variety of other flatfish (Fig. 3).

The structure of the pleurocidin prepropolypeptides bears certain resemblances to the frog dermaseptin precursors, which also contain a signal sequence of similar length (22 amino acids) and an acidic portion of 16-25 amino acids. From the full-length cDNA clone (Fig. 1A), the acidic portion of pleurocidin was shown to contain 21 residues. A major difference between the pleurocidin and dermaseptin prepolypeptides is the position of the acidic portion – downstream of the mature peptide in pleurocidin and upstream of the mature peptide in dermaseptins. The acidic proparts of defensins have been proposed to prevent interaction of the antimicrobial peptide with the membrane by neutralising the cationic charges (Valore et al. 1996) and this may also be its function in pleurocidin.

The signal sequences and acidic carboxy-terminal sequences of the pleurocidin family members are extremely highly conserved. The former, and possibly the latter, are presumed to target the precursor molecules to the cell membrane for secretion. Gene families for antimicrobial peptides that contain highly conserved signal peptides (often encoded by the first exon) followed by end products with different biological activities have been described from the dermaseptin family (Valore et al. 1996) and the GLa, xenopsin, levitide and caerulein, all of which are skin peptides from *Xenopus laevis* (Kuchler et al. 1989). These authors proposed that this modular gene structure allows targeting for secretion to be achieved for markedly different peptides using a common pathway. In the pleurocidin gene family, a modular structure is also present with exon 1 encoding the signal sequence and first half of the antimicrobial peptide, exon 2 encoding the next ten amino acids of the antimicrobial peptide, and exon 3 encoding the last three amino acids of the antimicrobial peptide and the acidic carboxy terminus.

The mature peptides encoded by WF2 and WF4 are 60% identical to each other (Fig. 6) and somewhat less similar to dermaseptin B1 (Amiche et al. 1994) and ceratotoxin B (Marchini et al. 1995). WF1 is 64% identical to WF1a but contains a

remarkably cationic stretch of 18 amino acids between the signal sequence and the mature peptide that is not present in WF1a. Whether or not this potentially antimicrobial 18-mer peptide arises when pleurocidin WF1 processing occurs remains to be determined. Both WF1 and WF1a contain an additional 10-11 amino acids relative WF2, WF3 and WF4 between the mature peptide and the acidic carboxy terminus. WF3 shares similarities with both WF2/4 and WF1/1a. Synthetic pleurocidin identical to the central portion of WF2 has been shown to protect Coho salmon against infection by *Vibrio anguillarum*, as have hybrid peptides based on pleurocidin, dermaseptin and ceratotoxin (Jia et al. 2000).

Our results of testing synthetic peptides against a variety of bacterial pathogens as well as the fungal pathogen, *Candida albicans*, show promising candidates with broad-spectrum antimicrobial activities. Of particular interest is the ability of the peptides NRC-13 and NRC-15 to inhibit the growth of methicillin-resistant *S. aureus* at concentrations as low as 4 μg/ml. NRC-13 is also capable of inhibiting the growth of *C. albicans* at 4 μg/ml, *P. aeruginosa* at 1 μg/ml (and killing *P. aeruginosa* at this concentration), and *A. salmonicida* at 2 μg/ml. This means that NRC-13 is highly active against a fish pathogen, a Gram-negative human bacterium, a drug-resistant Gram-positive human bacterium, and a yeast. The example of NRC-13 demonstrates the range of potential targets and applications for cationic antimicrobial peptides.

Our results also validate the process we used for selecting antimicrobially active peptides from a large amount of sequence data. The ability to accurately predict which peptides are likely to be active is a crucial link between genomics and therapeutics. While much work remains to be done in this area, we have clearly demonstrated that judicious application of the principles described earlier will aid in selecting active peptides.

The tissue-specific expression of the pleurocidin genes was assessed using northern blot analysis and RT-PCR. Northern analysis proved to be not sufficiently sensitive for detecting the low level of transcripts present in winter flounder mRNA. Transcripts were present only in skin in sufficient quantities to be detected by this method, so the more sensitive RT-PCR assay was used. Pleurocidin transcripts were found in both skin and intestine using this method, in agreement with the recently reported ultrastructural localisation of pleurocidin in these tissues (Cole, Darouiche et al. 2000) and supporting the role of pleurocidin in mucosal immunity. The transcript

size (approximately 200 bp) is consistent with the size of products obtained by RT-PCR (Table 6), showing that the pleurocidin genes are transcribed separately.

RT-PCR analysis showed that the genes for the different pleurocidin-like peptides are expressed in a tissue-specific manner with WF2 being expressed predominantly in the skin and gill and to a lesser extent in the muscle, intestine, stomach and liver whereas WF1 and WF4 are detected predominantly in the gill and skin (Fig. 7). WF3 and WFYT are expressed in most of the tissues sampled, WFX is detected solely in the skin and WF1a was not expressed in any of the tissues sampled. Possibly, the different antimicrobial peptides are required to control the growth of different bacterial populations in the two tissues. Since no RT-PCR products were detected for WF4, it is possible that this gene is expressed only at low levels in adult skin or intestine or that it is expressed at a different life stage or in a different tissue.

Using primers that did not discriminate between the transcripts of the various pleurocidin-like genes, expression was first detected at 5 dph and showed a progressive increase towards adulthood. However, recent experiments using primers specific for WF1, WF1a, WF2, WF3, WF4 , WFX and WFYT, transcripts were detected at different developmental stages (Fig. 9). WFX was only detectable at 20 dph, whereas WFYT, WF3 and WF2 were detectable at 5 dph and at higher levels between 25-36 dph. Interestingly, WF1 was not detectable at any larval stage and may only be expressed under specific environmental conditions in response to specific bacterial pathogens, as has been shown for *Drosophila* (Rivas and Ganz 1999). This is the first demonstration of developmental expression of an antimicrobial peptide in fish and shows that at least this component of innate immunity is present in early larval stages of winter flounder. Larval mortality prior to metamorphosis is of great concern and although the reasons for such mortality are not yet known, high bacterial load in the gut has been proposed (Padros, Minkoff et al. 1993). The adaptive immune systems of flatfish have been shown to develop later than those of other teleosts (Padros, Sala et al. 1991). Thus, the ability of larvae to produce antimicrobial peptides during this period may be crucial to survival, and the identification of factors that increase the production of such compounds would be of great benefit to aquaculturalists.

In conclusion, we have isolated a variety of cDNA and genomic sequences encoding the precursors of antimicrobial peptides identical to or similar to pleurocidin from a variety of flatfish species. Northern hybridisation and sequence analysis of RT-PCR products showed that expression was tissue-specific. Most importantly, the

timing of expression of different pleurocidin variants in developing larval winter flounder was determined, allowing an estimate of the onset of the innate immune system in this fish. Environmental parameters affecting the production of pleurocidin can now be assayed.

This work paves the way to further studies aimed at the over-expression of pleurocidin as a therapeutant for aquacultured fish and the production of disease-resistant fish through transgenic technology as has been demonstrated in transgenic tobacco expressing antimicrobial peptides (Jach et al. 1995) and proposed for fish (Jia et al. 2000). Furthermore, because many fish live in a saline environment, the properties of their antimicrobial peptides may be different from those produced by terrestrial animals and have application in unique situations. For instance, the pulmonary mucosa of patients with cystic fibrosis contain elevated NaCl concentrations, which inhibit the natural cationic peptides secreted by the lung (Goldman et al. 1997). Salt-adapted cationic peptides from marine fish may have application in the treatment of lung infections in these patients.

Hepcidins

Sequence analysis of one salmon EST (SL1-0412) and one halibut clone (Hb7.5), revealed the presence of unspliced transcripts and allowed the positions of some of the introns to be determined (Fig. 16). Similar to mouse, human and hybrid striped bass, the salmon hepcidin is composed of three exons and two introns (Park, Valore et al. 2001; Shike et al. 2002; Pigeon, Ilyin et al. 2001). The position of the first intron of salmon and bass are identical and correspond to a position two amino acids 5' to those of mouse and human. However, the second salmon intron and the second halibut intron of Hb7.5 correspond to a position two amino acids 3' to those of mouse and human and several amino acids 5' to that of the bass. This is probably due to "intron sliding" whereby the positions of introns have shifted by several nucleotides over the course of evolution. Interestingly, the deletion in WF4 corresponds precisely to the position of the first salmon intron and the second mouse/human intron, indicating an intermediate intron/exon structure.

Mouse contains two hepcidin genes that are clustered on the genome (Pigeon, Ilyin et al. 2001) but in human (Park, Valore et al. 2001) and striped bass (Shike et al. 2002) only one hepcidin gene has been identified. Although we have not determined

the number of hepcidin genes in winter flounder and Atlantic salmon, based on our research there are at least five in winter flounder, five in Atlantic halibut and four in Atlantic salmon. Since there are no *Sst*I sites within the hepcidin probe used in the Southern hybridization analysis, it is highly probable that the five winter flounder hepcidin genes reported here are clustered on two genomic fragments. Multiple genes for pleurocidin also exist (Douglas, Gallant et al. 2001) and are clustered on the genome (Fig. 11). Interestingly, all of the small flounders tested from the Atlantic exhibited a similar hybridizing band of 4.3 kb, indicating that they share similarity at the genomic level. Japanese flounder, found in the Pacific, exhibited a single hybridizing band of 5.5 kb.

The deduced amino acid sequences of the fish prepro-hepcidins can be aligned with those from mammals throughout their length but only show high similarity in the portion corresponding to the processed peptides (Fig. 17). However, within the fish, the signal peptide and the propiece are also very highly conserved. Conservation of these segments has also been noted in the pleurocidin family (Douglas, Gallant et al. 2001). The amino-termini of the processed peptides were assigned based on the amino acid sequence of human hepcidin (Krause, Neitz et al. 2000; Park, Valore et al. 2001) and the proximity to the RX(K/R)R motif characteristic of processing sites (Nakayama 1997). The molecular weights of the processed hepcidins from winter flounder and Atlantic salmon range from 1992 Da (WF2) to 3066 (WF1), comparable to hepcidins isolated from mouse, human and bass. With the exception of WF2, which has an acidic pI (5.54), the pIs of hepcidins are between 7.73 and 8.76.

Like pleurocidins, the amino acid sequences of the hepcidin variants are highly similar within species, suggesting relatively recent duplication of an ancestral gene. It is possible that the aquatic environment in which fish live necessitates the existence of a more diverse suite of antimicrobial peptides than in terrestrial mammals. In addition, this component of the innate immune system plays a more major role in fish than in mammals, which have a more highly evolved adaptive immune system.

The human hepcidin molecule has been proposed to form a secondary structure containing a series of β-turns, loops and distorted β-sheets (Park, Valore et al. 2001). Consensus secondary structure prediction of fish hepcidins show that they contain mostly random coil structure with some extended strand structure. With the exception of WF2, JFL6 and Hb357, all hepcidins reported thus far contain eight cys-

teine residues which are proposed to form four disulphide bonds (Krause, Neitz et al. 2000; Park, Valore et al. 2001) in the following linkage pattern: 1-4, 2-8, 3-7, 5-6 (Park, Valore et al. 2001). The loss of cysteine residues 1 and 3 from WF2 suggests that at least one disulphide bond cannot form.

Using gene-specific primers, we were able to demonstrate that different hepcidin genes are expressed in different tissues of both winter flounder (Fig. 20) and Atlantic salmon (Fig. 21). In Atlantic salmon, hepcidin was detectable in normal uninfected fish predominantly in liver, blood and muscle (Type I) and to a lesser extent in gill and skin (both types). This is consistent with the presence of three ESTs for Type I hepcidin in cDNA libraries constructed from uninfected livers, and the absence of ESTs for Type II hepcidin in cDNA libraries constructed from uninfected liver, spleen and head kidney. Type II hepcidin expression appears be confined to external epithelial surfaces in contact with the aqueous environment, whereas Type I hepcidin expression is more widespread, being expressed in liver, blood and muscle as well as external epithelial surfaces. In uninfected winter flounder, no transcripts of Type II hepcidin could be detected in any tissue but transcripts of Types I and III hepcidin were present in the liver and cardiac stomach. Type III hepcidin transcripts were also present in the esophagus.

Mouse hepcidin was also reported to be predominantly expressed in liver, and weakly in stomach, intestine, colon, lungs, heart and thymus by Northern analysis using one of the mouse hepcidin sequences as probe (Pigeon, Ilyin et al. 2001). However, this study did not discriminate between the two hepcidin genes and it is not known whether or not the two mouse genes are differentially expressed in tissues of mouse. Similarly, dot-blot analysis of human tissues and cell lines using the human hepcidin cDNA as probe revealed strong expression in adult and fetal liver and weaker expression in adult heart, fetal heart and adult spinal cord (Pigeon, Ilyin et al. 2001). An earlier study using RealTime quantitative RT-PCR (Krause, Neitz et al. 2000) revealed strong expression of hepcidin in human liver, heart and brain and weak expression in a variety of other tissues. Interestingly, we could not detect either Type I or Type II hepcidin expression in the brain of normal Atlantic salmon or winter flounder, or heart of normal Atlantic salmon. However, in infected animals, Type II hepcidin was expressed in both tissues, indicating that this form is the predominant one produced under conditions of stress.

It is intriguing that we detected transcripts of Type I hepcidin that were constitutively expressed in blood cells of Atlantic salmon. Constitutively expressed non-enzymic antimicrobial molecules have been reported only rarely in blood of fish; a small hydrophobic cationic peptide was found in mucus of rainbow trout (Smith et al., 2000) and moronecidin, an antimicrobial peptide from bass, was expressed in blood of uninfected animals (Lauth et al. 2002). Interestingly, expression of neither hepcidin increased in blood of infected salmon relative to the uninfected control animals. Possibly, hepcidin is fulfilling a role in iron homeostasis in control animals as well as an antimicrobial role. Its presence in circulating blood cells of uninfected animals may be a precautionary measure against impending infection.

Type I and II hepcidins from Atlantic salmon were up-regulated during infection with *Aeromonas salmonicida*, but to different extents in various tissues. While Type I hepcidin was noticeably up-regulated in the esophagus, stomach, pyloric caecae, liver, spleen, intestine, posterior kidney, rectum and muscle and to a lesser extent in anterior kidney and skin, Type II hepcidin showed a more dramatic increase in stomach, pyloric caecae, liver, spleen, intestine, brain, heart and muscle. Weaker up-regulation was present in esophagus, anterior and posterior kidney, skin and rectum. These results are consistent with those reported for bacterially challenged hybrid striped bass where up-regulation was most dramatic in liver, but was also demonstrated in skin, gill, intestine, spleen, anterior kidney and blood (Shike et al. 2002). It is not known whether there are multiple helpcidins in hybrid striped bass and, if so, whether they are differentially expressed as in Atlantic salmon and winter flounder.

Studies with mice have shown a 4.3-fold increase in hepcidin expression in livers of mice injected with LPS and a 7-fold increase in primary hepatocytes exposed to LPS (Pigeon, Ilyin et al. 2001). These studies were based on Northern analysis using only one of the mouse hepcidin sequences as probe, and were therefore unable to distinguish possible differential expression of the two mouse variants. Similar increases were noted in livers of mice subjected to iron overload, but not for primary hepatocytes exposed to iron citrate, possibly due to the differentiation status of the cultured hepatocytes. The fact that both iron overload and LPS exposure increase hepcidin expression indicates the importance of these two factors in the host response to pathogens.

During infection, iron is removed from the system by various mechanisms so that it is unavailable for use by invading pathogens. It has been proposed that recently

discovered transferrin receptor2 mediates iron uptake by hepatocytes and increases their expression of hepcidin (Fleming and Sly 2001; Nicolas, Bennoun et al. 2001). Hepcidin, in turn, increases iron accumulation in macrophages and increases dietary iron absorption in duodenal crypt cells via β2 microglobulin, HFE and transferrin receptor1. These crypt cells differentiate into enterocytes with reduced amounts of iron transport proteins, thereby decreasing dietary iron uptake. Hepcidin thus appears to play a crucial role in iron homeostasis during inflammation as well as acting as an antimicrobial peptide. It is also possible that hepcidin could modulate expression of liver-derived acute phase proteins and exhibit synergistic effects with other components of the immune system.

Antimicrobial peptides have been shown to modulate gene expression in mouse macrophages (Scott, Rosenberger et al. 2000), and it is possible that they may exert similar effects in fish macrophages or hepatocytes. The presence of a functional nuclear localization signal (four K/R residues in a row) within prohepcidin of mouse and human indicates that hepcidin could act as a signaling molecule involved in maintenance of iron homeostasis in these organisms (Pigeon, Ilyin et al. 2001). Interestingly, the nuclear localization signal also contains the recognition signal for processing of prohepcidin, indicating that nuclear localization would occur only prior to removal of the propiece, or that the propiece itself is localized to the nucleus. Teleost hepcidins contain only 3 out of 4 K/R residues, which may not be sufficient for nuclear localization; a role for hepcidin in intracellular signaling awaits testing with synthetic or *in vitro*-expressed peptide.

In conclusion, we have presented the sequences of fourteen new hepcidin-like peptides from Atlantic salmon, Atlantic halibut and winter flounder and demonstrated the presence of related sequences in several flatfish species by Southern hybridization. Furthermore, we have shown that the various types of fish hepcidins are differentially expressed in a tissue-specific manner in normal fish, as a result of bacterial infection, and during larval development. Apparently in fish, different tissues produce hepcidins in a constitutive or inducible manner, indicating that hepcidin variants may have different functions under different circumstances. Given their role in iron homeostasis in mammals, it is possible that fish hepcidin variants may fulfil this role as well as that of killing specific pathogens. *In vitro* expression of hepcidin variants will allow their

spectrum of antimicrobial activity to be determined as well as their effect on the innate immune response.

References

Amiche, M., F. Ducanel, et al. (1994). "Precursors of vertebrate peptide antibiotics dermsaseptin B and adenoregulin have extensive sequence identities with precursors of opioid peptides dermorphin, dermenkephalin, and deltorphins. J. Biol. Chem. 269: 17847-52.

Amsterdam, D. 1996. Susceptibility Testing of Antimicrobials in Liquid Media. *In* V. Lorian (ed.), Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore.

Casteels-Jossen, K., T. Capaci, et al. (1993). "Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response." EMBO J. 12: 1569-78.

Cohen, S., M. Skiguchi, J. Stern, and H. Barner. 1963. The synthesis of messenger RNA without protein synthesis in normal and phage-infected thymineless strains of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A Biochem. 49:699-706.

Cole, A. M., R. O. Darouiche, et al. (2000). "Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity." Antimic. Ag Chemotherapy. 44: 2039-45.

Cole, A. M., P. Weis, et al. (1997). "Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder." J. Biol. Chem. 272(18): 12008-12013.

Douglas, S. E., C. E. Bullerwell, et al. (1999). "Molecular investigation of aminopeptidase N expression in the winter flounder, *Pleuronectes americanus*." J. Appl. Ichthyol. 15: 80-86.

Douglas, S. E., J. W. Gallant, et al. (1999). "Winter flounder expressed sequence tags: establishment of an EST database and identification of novel fish genes." Mar. Biotechnol. 1: 458-464.

Douglas, S. E., J. W. Gallant, et al. (1998). "Isolation of cDNAs for trypsinogen from the winter flounder, *Pleuronectes americanus*." J. Mar. Biotechnol. 6: 214-9.

Douglas, S. E., J. W. Gallant, et al. (2001). "Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter flounder, *Pleuronectes americanus* (Walbaum)." Dev. Comp. Immunol. 25: 137-147.

Douglas, S. E., A. Gawlicka, et al. (1999). "Ontogeny of the stomach in winter flounder: characterisation and expression of the pepsinogen and proton pump genes and determination of pepsin activity." J. Fish Biol. 55: 897-915.

Douglas, S. E., S. C. M. Tsoi, et al. (2002). Expressed sequence tags - a snapshot of the fish genome. A Step Toward the Great Future of Aquatic Genomics, Tokyo, Japan.

Fleming, R. E. and W. S. Sly (2001). "Hepcidin: A putative iron-regulatory hormone relevant to hereditary hemochromatosis and the anemia of chronic disease." Proc. Natl. Acad. Sci. USA 98(15): 8160-8162.

Garvan, J. (1996). SeqVu. Sydney, Australia, The Garvan Institute of Medical Research.

Goldman, M. J., G. M. Anderson, et al. (1997). "Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis." Cell. 88: 553-60.

Gong, Z., K. V. Ewart, et al. (1996). "Skin antifreeze protein genes of the winter flounder, *Pleuronectes americanus*, encode distinct and active polypeptides without the secretory signal and prosequences." J. Biol. Chem. 271: 4106-12.

Gudmundsson, G. H., D. A. Lidholm, et al. (1991). "The cecropin locus. Cloning and expression of a gene cluster encoding three antibacterial peptides in Hyal*ophora cecropla*." J. Biol. Chem. 166: 11510-7.

Hancock, R. E. W. and R. Lehrer (1998). "Cationic peptides: a new source of antibiotics." TIBTECH 16: 82-88.

Hwang, E.-Y., J.-K. Seo, et al. (1999). "Purification and characterization of a novel antimicrobial peptide from the skin of the hagfish, *Eptatretus burgeri*." J. Food Sci. Nutr. 4(1): 28-32.

Jach, G., B. Gornhardt, et al. (1995). "Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco." Plant J. **8**: 97-109.

Jia, X., A. Patrzykat, et al. (2000). "Antimicrobial peptides protect coho salmon from *Vibria anguillarium* infections." Appl. Environ. Microbiol. **66**: 1928-32.

Krause, A., S. Neitz, et al. (2000). "LEAP-1, a novel highly disulfide-bonded human peptide, exhibits antimicrobial activity." FEBS Lett. **480**: 147-150.

Kuchler, K., G. Kreil, et al. (1989). "The genes for the frog skin peptides GLAa, xexopsin, levitide, and caerulin contain a homologous export exon encoding a signal sequence and part of an amphiphilic peptide." Eur. J. Biochem. **179**: 281-5.

Lauth, X., H. Shike, et al. (2002). "Discovery and characterization of two isoforms of moronecidin, a novel antimicrobial peptide from hybrid striped bass." J. Biol. Chem. **277**: 5030-5039.

LeMaitre, C., N. Orange, et al. (1996). "Characterization and ion channel activities of novel antibacterial proteins from the skin mucosa of carp (*Cyprinus carpio*)." Eur. J. Biochem. **240**: 143-149.

Marchini, D., A. G. O. Manetti, et al. (1995). "cDNA sequence and expression of the certotoxin gene ecoding an antibacteiral sex-specific peptide from the dedfly *Ceratitis capitata* (diptera). J. Biol. Chem. **270**: 6199-204.

Marck, C. (1992). DNA Strider Version 1.2. Service de Biochimie - Bat 142, Centre d'Etudes Nucleares de Saclay,. Gif-sur-Yvette, France.

Moore, K. S., S. Wehrli, et al. (1993). "Squalamine: an aminosterol antibiotic from the shark." Proc. Natl. Acad. Sci. USA. **90**: 1354-1358.

Nakayama, K. (1997). "Furin: a mammalian subtilisin/Kex2p-like endoprotease involved in processing of a wide variety of precursor proteins." Biochemical J. **327**: 625-635.

Nicolas, G., M. Bennoun, et al. (2001). "Lack of hepcidin gene expression and severe tissue iron overload in upstream stimulatory factor 2 (USF2) knockout mice." Proc. Natl. Acad. Sci. USA. **98**(15): 8780-8785.

60409422 .092202

Oren, Z. and Y. Shai (1996). "A class of highly potent antibacterial peptides derived from pardaxin, a pore-forming peptide isolated from Moses sole fish *Pardachirus marmoratus*." Eur. J. Biochem. **237**(1): 303-310.

Padros, F., G. Minkoff, et al. (1993). "Histopathological events throughout the development of turbot (*Scophthalmus maximus* L.)." J. Comp. Pathol. **109**: 321-4.

Padros, F., R. Sala, et al. (1991). Organogenesisin turbot, *Scophthalmus maximus*, larvae related to the main developmental stages: in Larvi'91. Fish and Crustacean Larviculture Symposium. Ghent, Belgium: European Aquaculture Society.

Park, C. B., J. H. Lee, et al. (1997). "A novel antimicrobial peptide from the loach, Misgurnus anguillicandatus." FEBS Lett. **411**: 173-178.

Park, C. H., E. V. Valore, et al. (2001). "Hepcidin, a urinary antimicrobial peptide synthesized in the liver." J. Biol. Chem. **276**(11): 7806-7810.

Park, I. Y., C. B. Park, et al. (1998). "Parasin I, an antimicrobial peptide derived from histone H2A in the catfish, *Parasilurus asotus*." FEBS Lett. **437**(3): 258-262.

Pigeon, C., G. Ilyin, et al. (2001). "A new mouse liver-specific gene, encoding a protein homologous to human antimicrobial peptide hepcidin, is overexpressed during iron overload." J. Biol. Chem. **276**(11): 7811-7819.

Rivas, L. and T. Ganz. (1999). "Eukaryotic antibiotic peptides: not only a membrane business." Drug Discovery Today. **4**: 254-6.

Scott, M. G., C. M. Rosenberger, et al. (2000). "An a-helical cationic antimicrobial peptide selectively modulates macrophage responses to lipopolysaccharide and directly alters macrophage gene expression." J. Immunol. **165**: 3358-3365.

Shike H, Lauth X, Westerman ME, Ostland VE, Carlberg JM, Van Olst JC, Shimizu C, Burns JC (2002). "Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge." Eur J Biochem; **269**:2232-2237.

Silphaduang, U. and E. J. Noga (2001). "Peptide antibiotics in mast cells of fish." Nature **414**: 268-9.

Smith, V. J., J. M. O. Fernandes, et al. (2000). "Antibacterial proteins in rainbow trout, *Oncorhynchus mykiss*." Fish Shellfish Immunol. **10**: 243-260.

Thompson, J., D. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice." Nucleic Acids Res 22: 4673-4680.

Trust T. J,. Ishiguro, E. E., Chart, H. and Kay W. W. (1983) Virulence properties of *Aeromonas salmonicida*. J. World Maricul. Soc.14:193-200.

Valore, E. V., E. Martin, et al. (1996). "Intramolecular inhibition of human defensin HNP-1 by its propiece." J. Clin. Invest. 97: 1624-9.

Wu, M., E. Maier, R. Benz, and R. E. W. Hancock. 1999. Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochem. 38:7235-7242.

ABSTRACT

The invention disclosed relates to antimicrobial peptides. Antimicrobial peptides play a crucial role as the first line of defense against invading pathogens. Several types of antimicrobial peptides have been isolated from fish, mostly of the cationic α-helical variety. The cDNA sequences of fourteen highly disulphide-bonded hepcidin-like peptides have been isolated from winter flounder, *Pleuronectes americanus*, Atlantic halibut, *Hippoglossus hippoglossus,* and Atlantic salmon, *Salmo salar.* These hepcidins consist of a 24 amino acid signal peptide and an acidic propiece of 38-40 amino acids in addition to the mature processed peptide of 19-27 amino acids. The sequences of numerous pleurocidins isolated from different fish species is also described.

A.

PL1 primer

PL5' primer

acaaagcccactttgtattcgcaagaATGAAGTTCACTGCCACCTTCCTCATGATGGCCATCTTCGTCCTC

M K F T A T F L M M A I F V L

ATGGTTGAACCTGGAGAGTGTGGCTGGGGAAGCTTTTTTAAAAAGGCTGCTCACGTTGGCAAGCATGTTGGC

M V E P G E C G W G S F F K K A A H V G K H V G

PL3' primer

AAGGCGGCCCTTACTCATTACCTTGGCGATAAGCAGGAGCTCAACAAGCGTGCAGTCGATGAAGACCCAAAT

K A A L T H Y L G D K Q E L N K R A V D E D P N

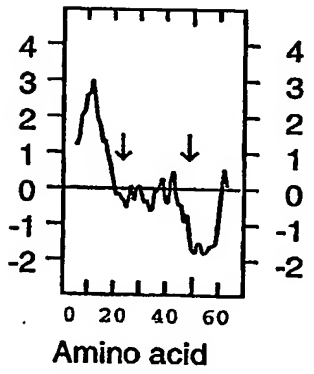
PL2 primer

GTTATTGTTTTTGAATGAagaaatcgccttgaaggagccttcagatgatatataatccttcttgcttttaatgaaataaatcagacttttacctgcaa

V I V F E *

cagcaaaaaaaaaaaaaaaaa

B.



C.

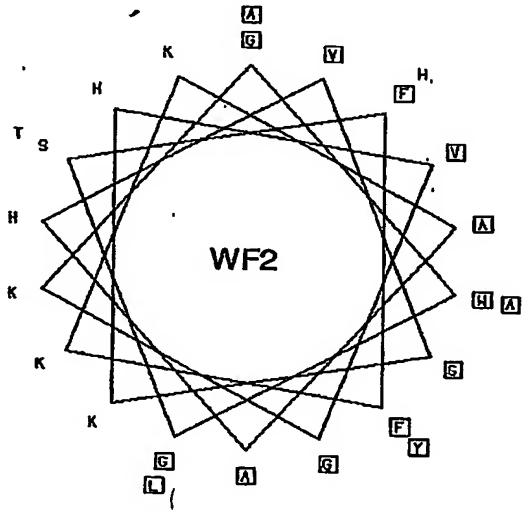


Figure 1

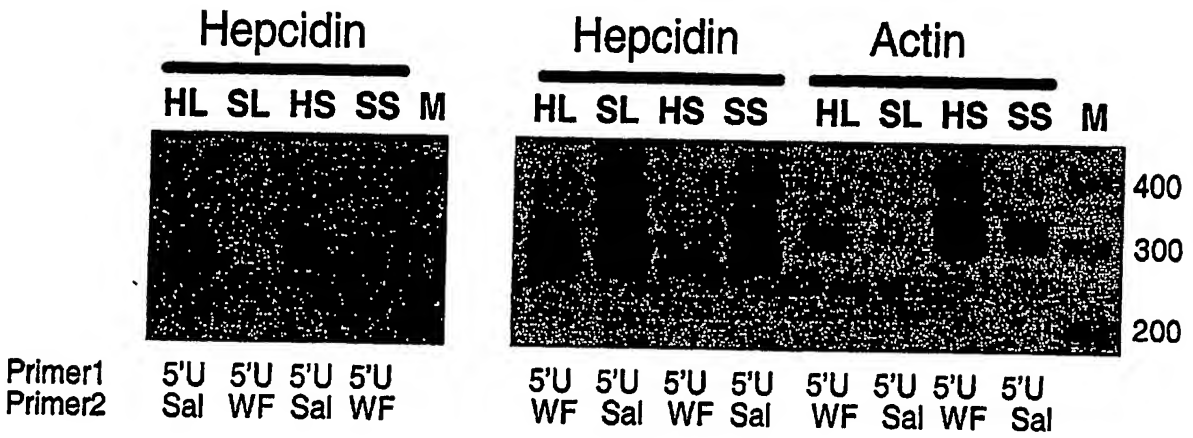
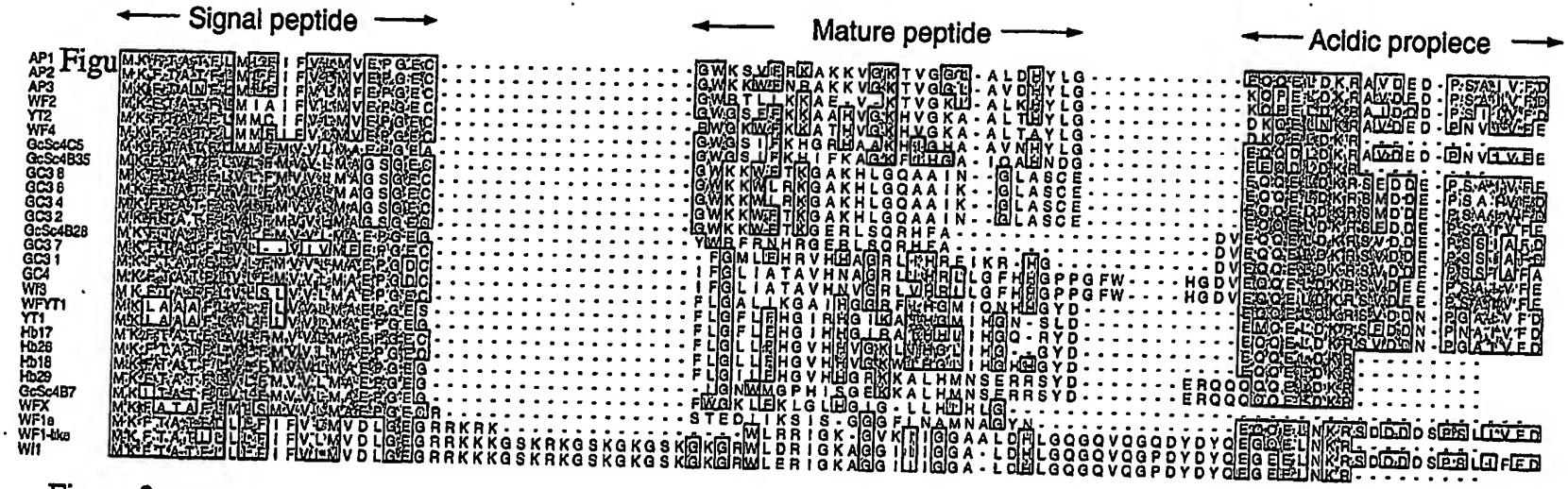

Hepcidin
Hepcidin
Actin
HL SL HS SS M
HL SL HS SS
HL SL HS SS M
400
300
200
Primer1
Primer2
5'U Sal
5'U WF
5'U Sal
5'U WF
5'U WF
5'U Sal
5'U WF
5'U Sal
5'U WF
5'U Sal
5'U WF
5'U Sal
5'U WF
5'U Sal
5'U WF
5'U Sal

Figure 2



Figu

Figure 3

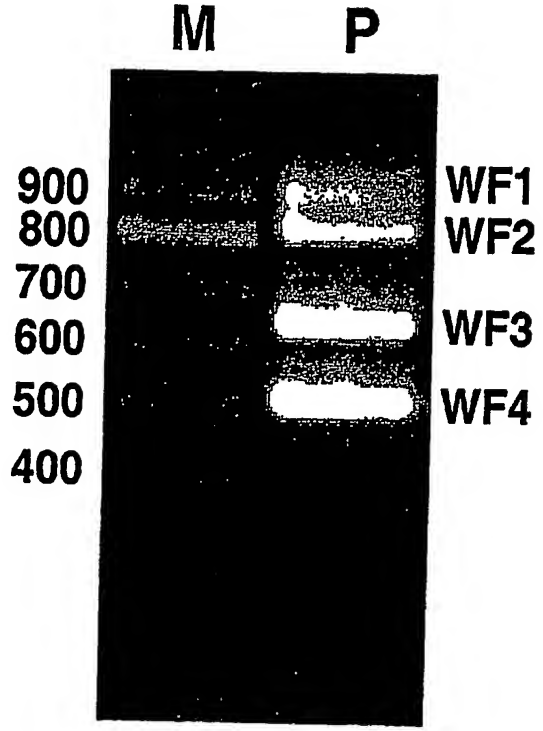


Figure 4

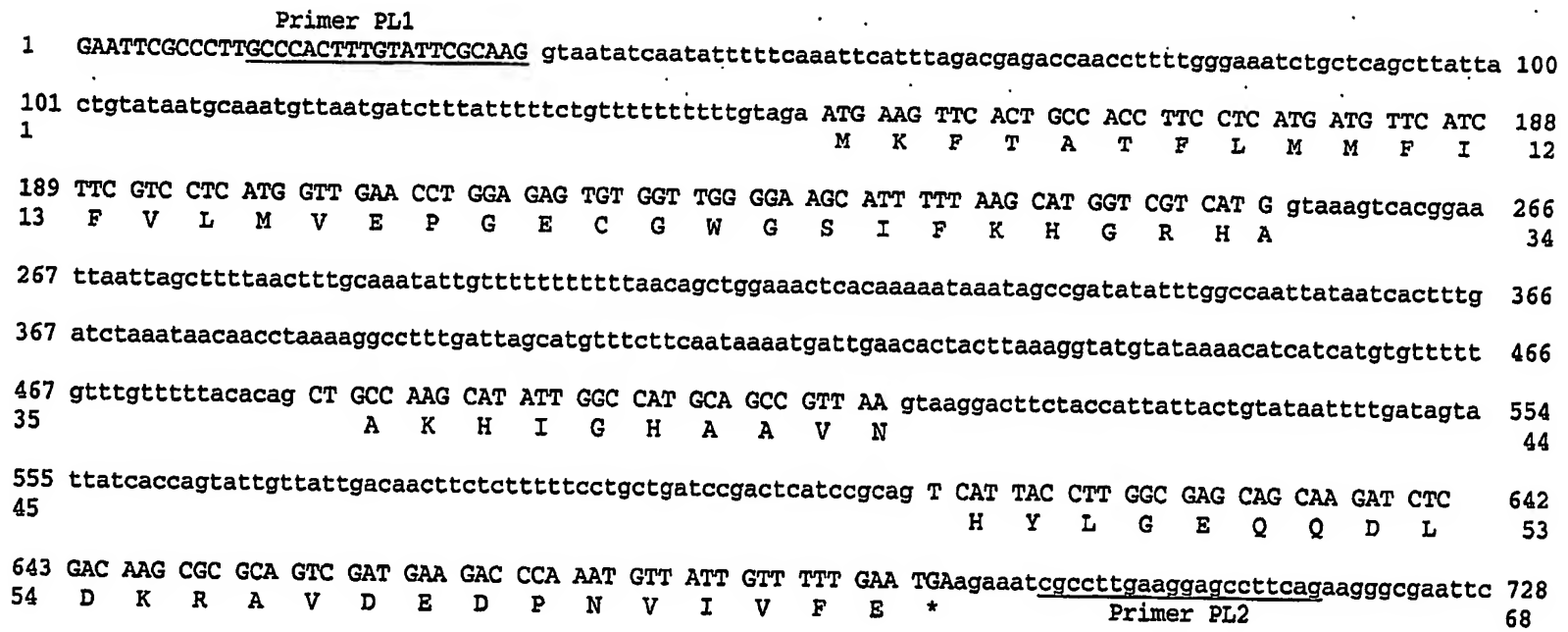

Primer PL1
1 GAATTCGCCCTTGCCCACTTTGTATTCGCAAG gtaatatcaatatttttcaaattcatttagacgagaccaacctttgggaaatctgctcagcttatta 100
101 ctgtataatgcaaatgttaatgatctttatttttctgtttttttttttgtaga ATG AAG TTC ACT GCC ACC TTC CTC ATG ATG TTC ATC 188
1 M K F T A T F L M M F I 12
189 TTC GTC CTC ATG GTT GAA CCT GGA GAG TGT GGT TGG GGA AGC ATT TTT AAG CAT GGT CGT CAT G gtaaagtcacggaa 266
13 F V L M V E P G E C G W G S I F K H G R H A 34
267 ttaattagcttttaactttgcaaatattgttttttttttttaacagctggaaactcacaaaaataaatagccgatatatttggccaattataatcactttg 366
367 atctaaataacaacctaaaaggcctttgattagcatgtttcttcaataaaatgattgaacactacttaaaggtatgtataaaacatcatcatgtgttttt 466
467 gtttgtttttacacag CT GCC AAG CAT ATT GGC CAT GCA GCC GTT AA gtaaggacttctaccattattactgtataatttttgatagta 554
35 A K H I G H A A V N 44
555 ttatcaccagtattgttattgacaacttctctttttcctgctgatccgactcatccgcag T CAT TAC CTT GGC GAG CAG CAA GAT CTC 642
45 H Y L G E Q Q D L 53
643 GAC AAG CGC GCA GTC GAT GAA GAC CCA AAT GTT ATT GTT TTT GAA TGAagaaatcgccttgaaggagccttcagaagggcgaattc 728
54 D K R A V D E D P N V I V F E * Primer PL2 68

Figure 5

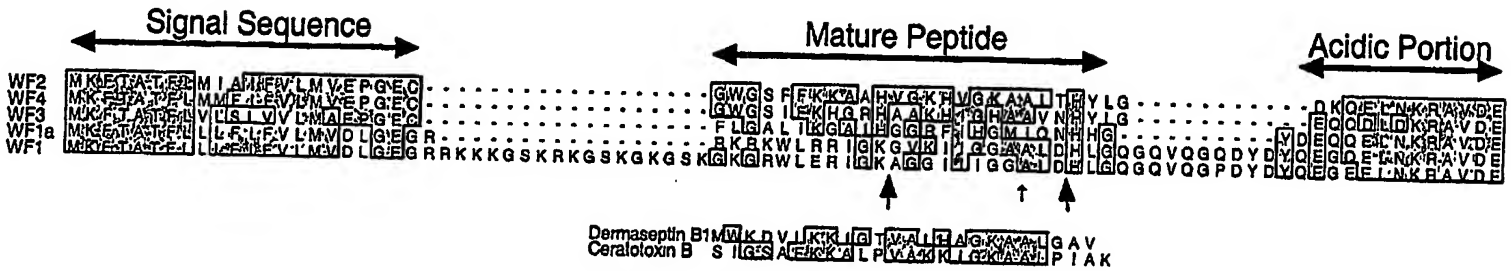

Signal Sequence
Mature Peptide
Acidic Portion
WF2
WF4
WF3
WF1a
WF1
Dermaseptin B1
Ceratotoxin B

Figure 6

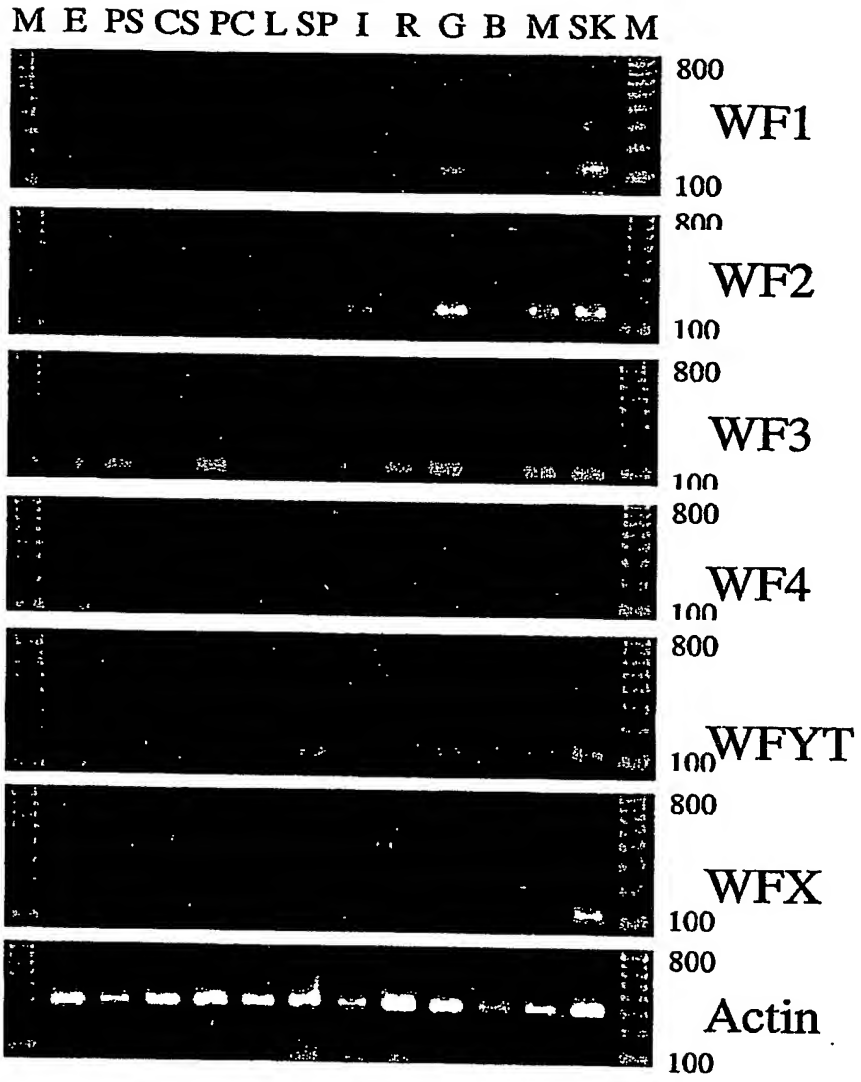

M E PS CS PC L SP I R G B M SK M
800
WF1
100
800
WF2
100
800
WF3
100
800
WF4
100
800
WFYT
100
800
WFX
100
800
Actin
100

Figure 7

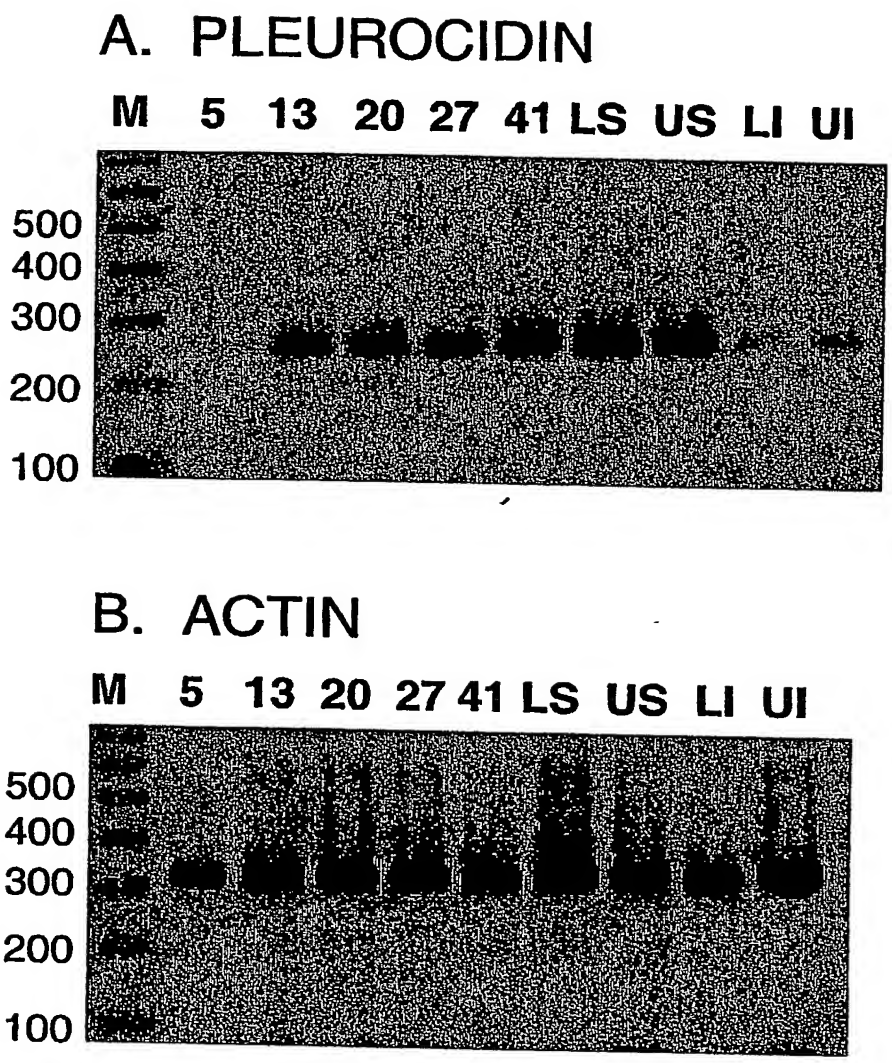

A. PLEUROCIDIN
M 5 13 20 27 41 LS US LI UI
500
400
300
200
100
B. ACTIN
M 5 13 20 27 41 LS US LI UI
500
400
300
200
100

Figure 8

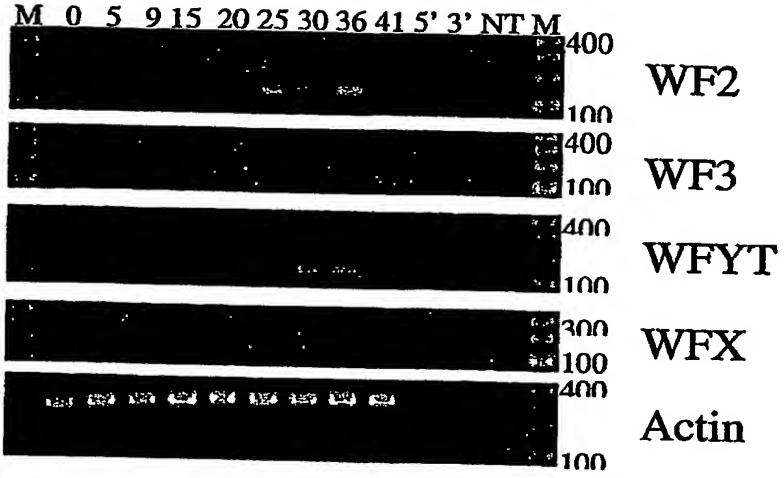

M 0 5 9 15 20 25 30 36 41 5' 3' NT M
400
100
WF2
400
100
WF3
400
100
WFYT
300
100
WFX
400
100
Actin

Figure 9

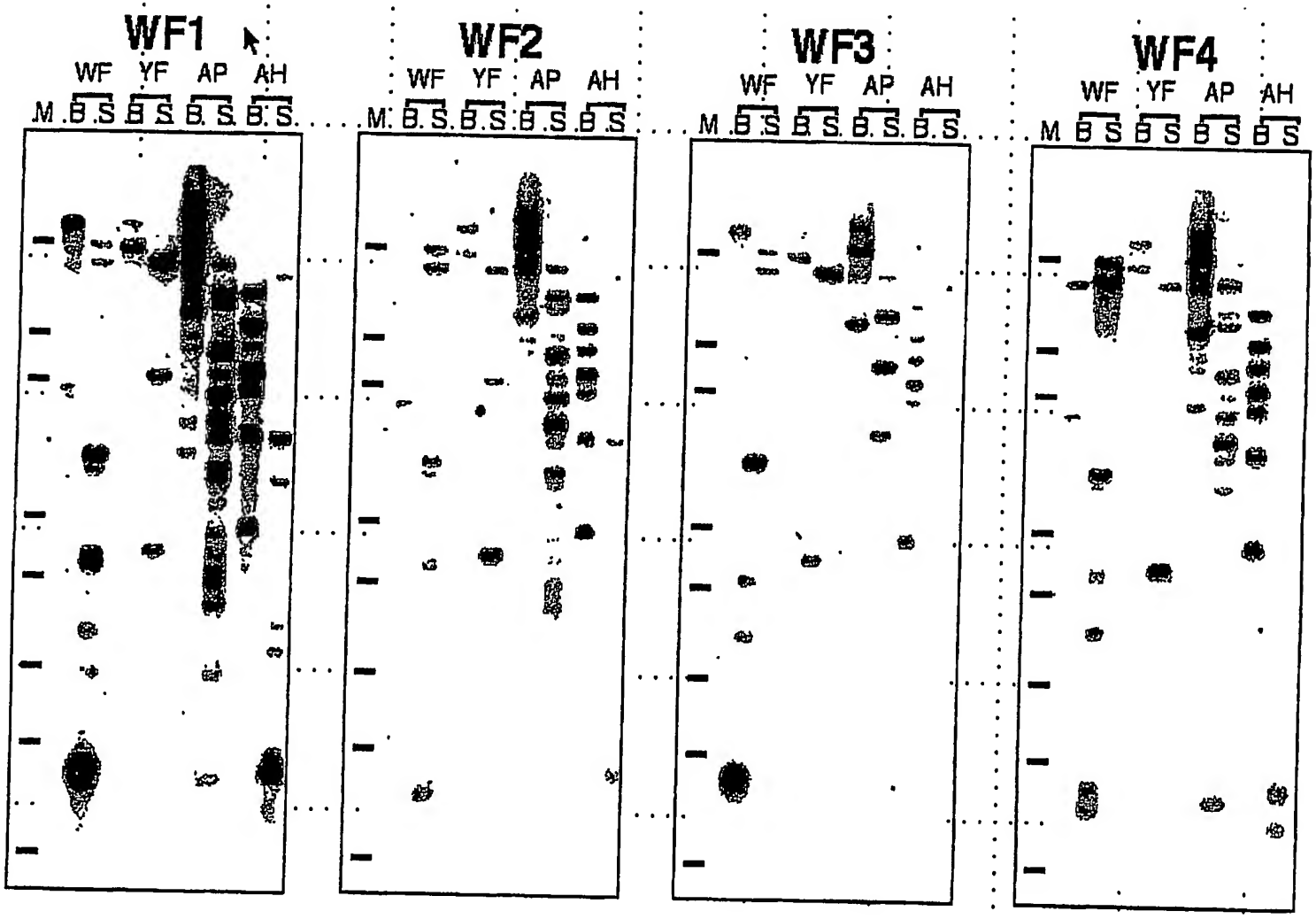
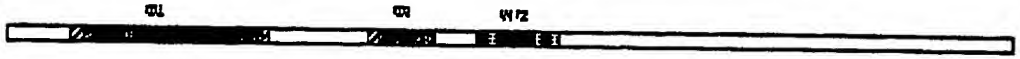


Figure 10



Lambda 5.1 (12.5 Kb)

Lambda 1.1 (17.5 Kb)

Figure 11

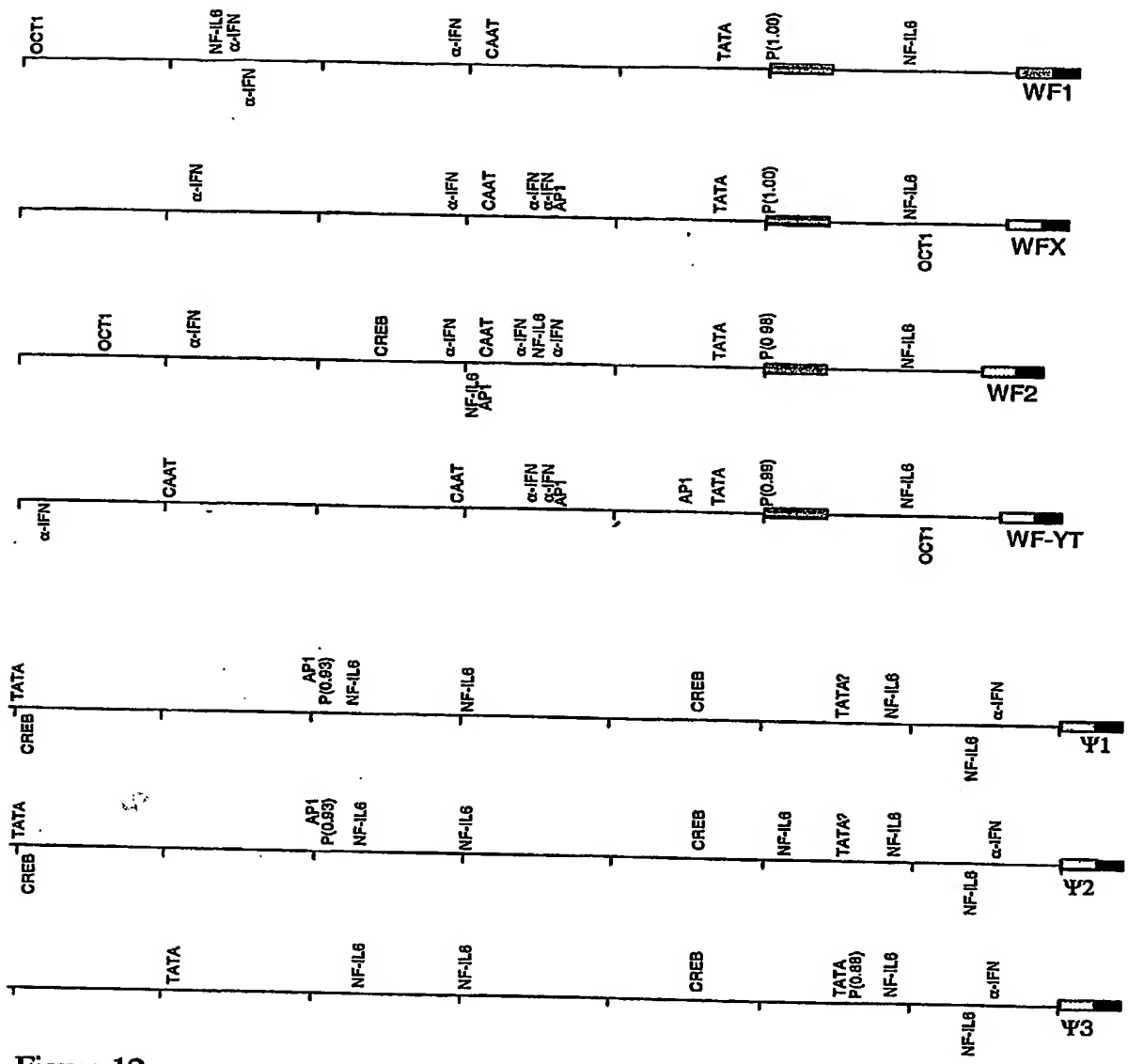

OCT1
NF-IL6
α-IFN
α-IFN
α-IFN
CAAT
TATA
P(1.00)
NF-IL6
WF1
α-IFN
α-IFN
CAAT
α-IFN
AP1
TATA
P(1.00)
NF-IL6
OCT1
WFX
OCT1
α-IFN
CREB
α-IFN
CAAT
α-IFN
NF-IL6
α-IFN
TATA
P(0.98)
NF-IL6
NF-IL6
AP1
WF2
α-IFN
CAAT
CAAT
α-IFN
AP1
AP1
TATA
P(0.98)
NF-IL6
OCT1
WF-YT
TATA
CREB
AP1
P(0.93)
NF-IL6
NF-IL6
CREB
TATA?
NF-IL6
α-IFN
NF-IL6
Ψ1
TATA
CREB
AP1
P(0.93)
NF-IL6
NF-IL6
CREB
NF-IL6
TATA?
NF-IL6
α-IFN
NF-IL6
Ψ2
TATA
NF-IL6
NF-IL6
CREB
TATA
P(0.88)
NF-IL6
α-IFN
NF-IL6
Ψ3

Figure 12

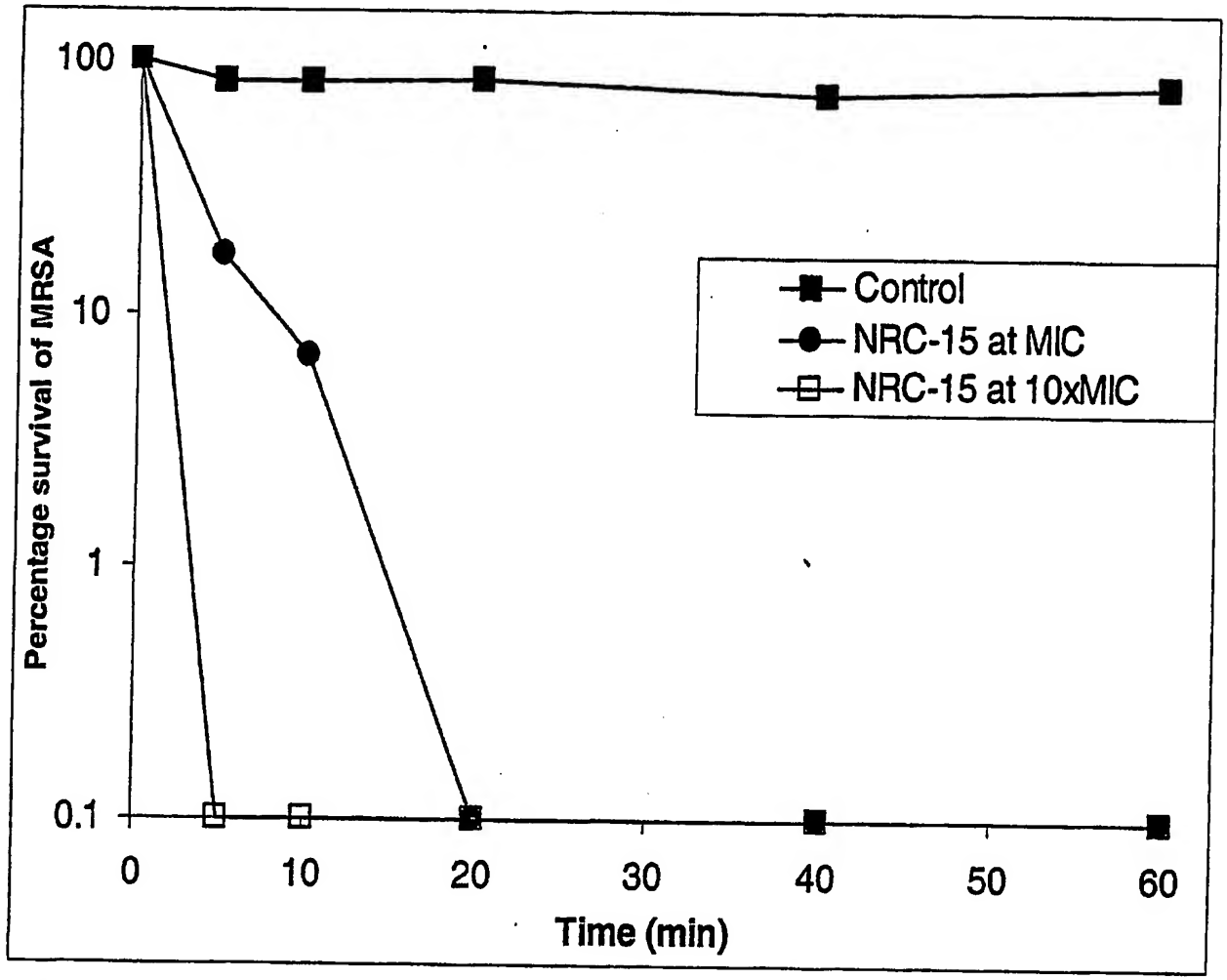

100
10
1
0.1
Percentage survival of MRSA
0
10
20
30
40
50
60
Time (min)
Control
NRC-15 at MIC
NRC-15 at 10xMIC

Figure 13

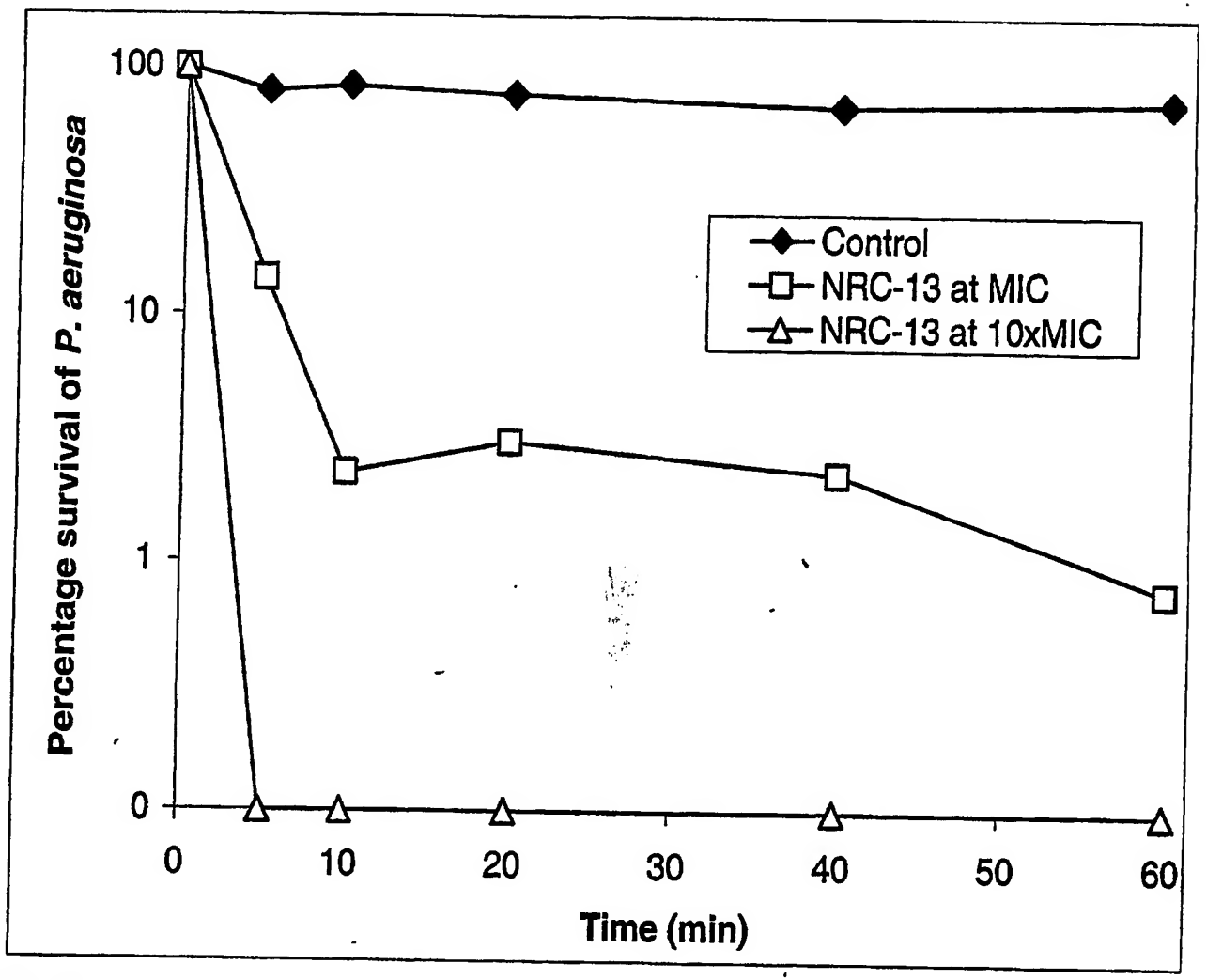

Percentage survival of P. aeruginosa
100
10
1
0
0
10
20
30
40
50
60
Time (min)
Control
NRC-13 at MIC
NRC-13 at 10xMIC

Figure 14

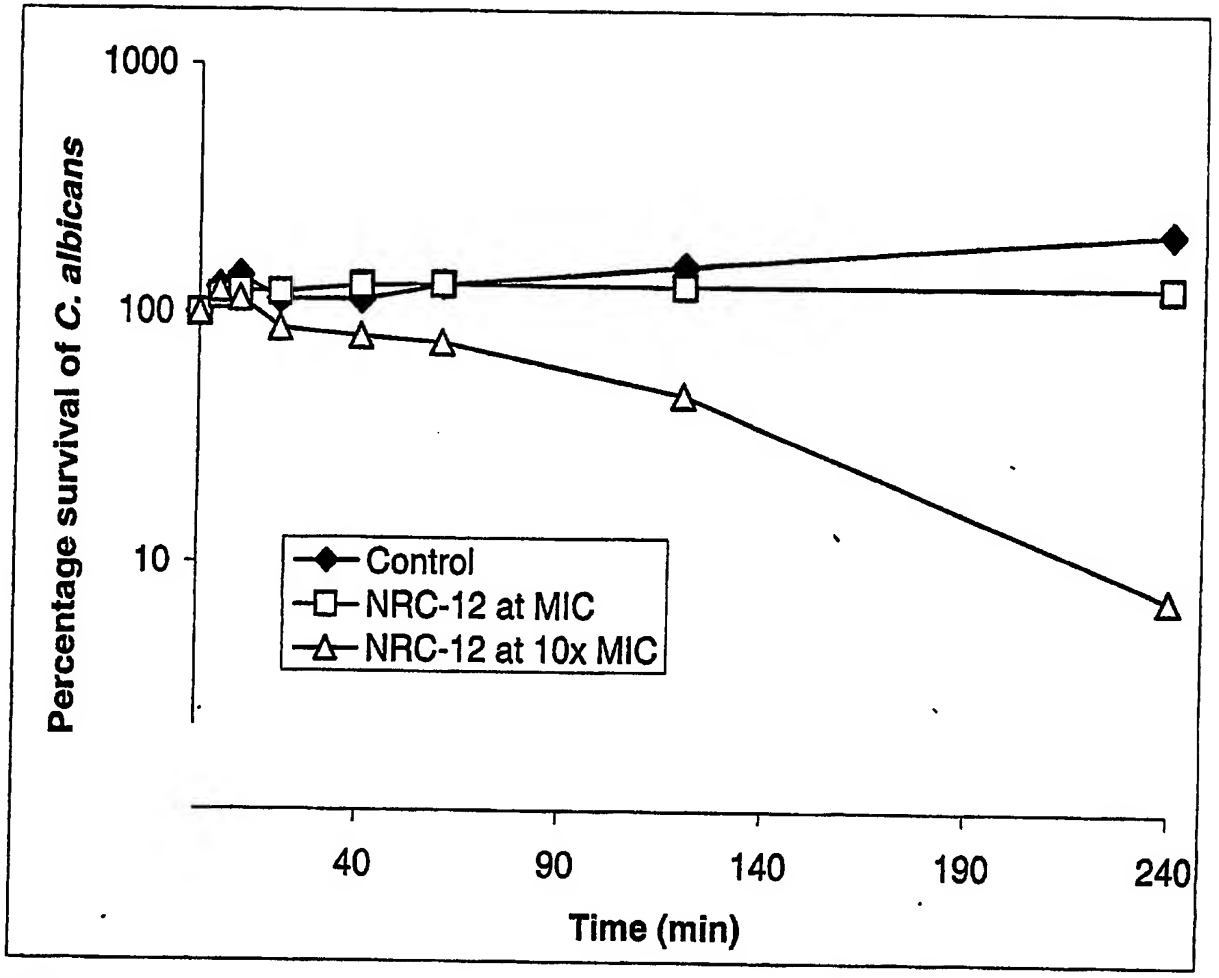

1000
100
10
Percentage survival of C. albicans
Control
NRC-12 at MIC
NRC-12 at 10x MIC
40
90
140
190
240
Time (min)

Figure 15

A.

```
1   ctgacaccaaaagaacaatcaatcaactttggactcgtcttagtgcattgaaaattgtgcgttggagagcgtcgctttt
81  gggaacattgaagagttctgatcttcctcataaactgtcacttcaatttcaactgatttcaacaggactttaaataggc
161 tataaacttcctaaaaaaaacgaga ATG AAG GCC TTT AGT GTT GCA GTG GTA CTC GTC ATT GCA
1                             M   K   A   F   S   V   A   V   V   L   V   I   A
225 TGT ATG TTC ATC CTT GAA AGC ACC GCT GTT CCT TTC TCC GAG gtatgtccaattctccaacaccaa
14  C   M   F   I   L   E   S   T   A   V   P   F   S   E
291 cccactacaaacatgtgtgcatcgattttagaggttggtcatgactcatttgtgcctaatgtctttgcag GTG CGA
28                                                                          V   R
367 ACG GAG GAG GTT GGA AGC TTT GAC AGT CCA GTT GGG GAA CAT CAA CAG CCG GGC GGC GAG
30  T   E   E   V   G   S   F   D   S   P   V   G   E   H   Q   Q   P   G   G   E
427 TCC ATG CAT CTG CCG gtacgttcaattgaatgaatgaattacgctaattacctttagcaaattaacatttagtg
50  S   M   H   L   P
502 gttgcgttttacccctcggaatagaattagatcagtagcgctagctgttaaccatttgattgtgagccgttagagggcttc
582 aggggagcagtgtgcaacgtggttgtgaagtggagatatacttacttgcttgttccctccttttttcatattattttct
662 tagcggggatacag GAG CCT TTC AGG TTC AAG CGT CAG ATC CAC CTC TCC CTG TGC GGT TTG
55                 E   P   F   R   F   K   R   Q   I   H   L   S   L   C   G   L
724 TGC TGC AAC TGC TGT CAC AAC ATT GGC TGT GGC TTT TGC TGC AAA TTC TAA ggacctgcccgc
71  C   C   N   C   C   H   N   I   G   C   G   F   C   C   K   F   *
787 aacatttctagtttgtacatgtttgcaatgttttctttctgagatgttgttttgtgactatgataatgatttataaaa
867 tcacttcttattgtgaacttttaaaaaaataaacacattctttgaataacaaaaaaaaaaaaaaaaaa
```

B.

```
1   cgcccttaag ATG AAG ACA TTC AGT GTT GCA GTT GCA GTG GTG GTC GTC CTC GCA TGT ATG
1              M   K   T   F   S   V   A   V   A   V   V   V   V   L   A   C   M
62  TTC ATC CTT GAA AGC ACC GCT GTT CCT TTC TCC GAG GTG CGA ACG GAG GAG GTT GAA AGC
18  F   I   L   E   S   T   A   V   P   F   S   E   V   R   T   E   E   V   E   S
122 ATT GAC AGT CCA GTT GGG GAA CAT CAA CAG CCG GGC GGC ACG TCC ATG AAT CTG CCG gtac
38  I   D   S   P   V   G   E   H   Q   Q   P   G   G   T   S   M   N   L   P
183 gttcaatttagtgaatgaattaagtaattacctttagcaaattaacatctaagtggttgcgttttcacccttggaattgaa
263 ttagccccactagcgctagttgttaaccatttgattgtgagccggtagagagggcttcagggcgagtagtgtgaatacttg
343 tgaagtggagacttggacaaaaatacttaccatgtgcttgttccccaccttttcattttcttttcttggctgagatacag
423 ATG CAT TTC AGG TTC AAG CGT CAG AGC CAC CTC TCC CTG TGC CGT TGG TGC TGC AAC TGC
57  M   H   F   R   F   K   R   Q   S   H   L   S   L   C   R   W   C   C   N   C
483 TGT CAC AAC AAG GGC TGT GGC TTC TGC TGC AAA TTC TGA ggacctgcccagcaaagggcgaatcagtt
77  C   H   N   K   G   C   G   F   C   C   K   F   *
558 taaaacac
```

C.

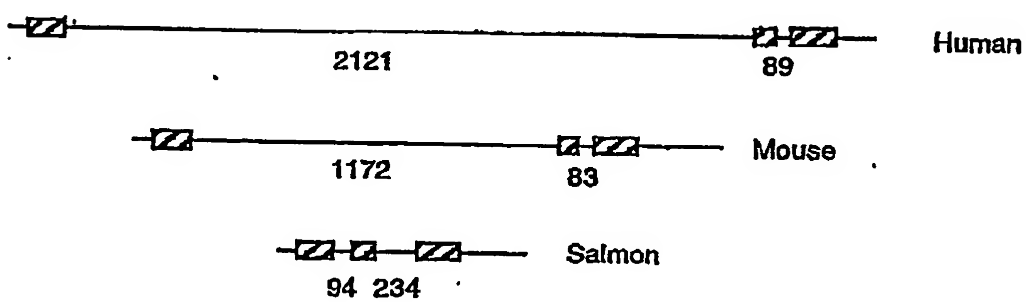


Fig. 16

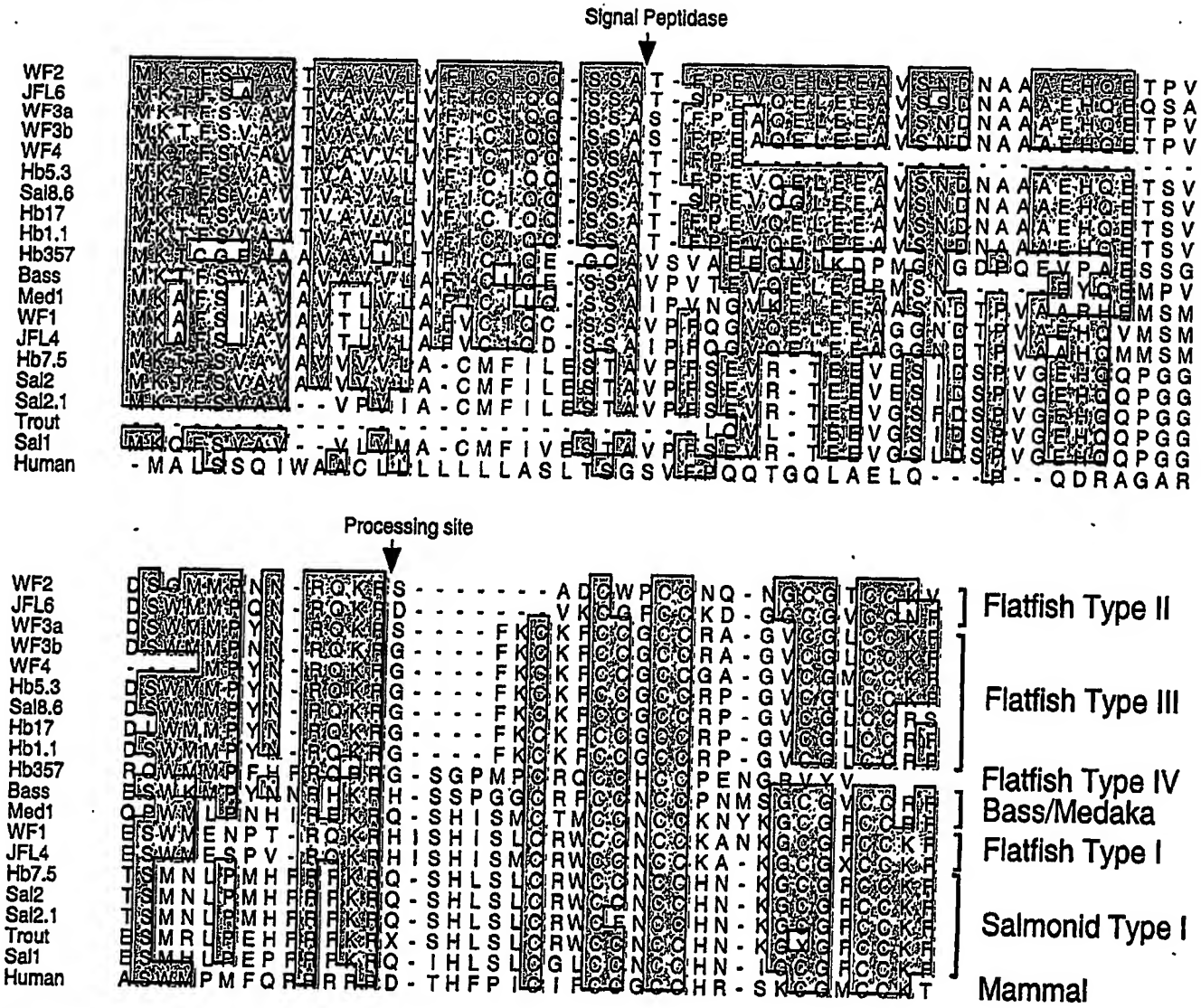

Signal Peptidase
Processing site
WF2
JFL6
WF3a
WF3b
WF4
Hb5.3
Sal8.6
Hb17
Hb1.1
Hb357
Bass
Med1
WF1
JFL4
Hb7.5
Sal2
Sal2.1
Trout
Sal1
Human
Flatfish Type II
Flatfish Type III
Flatfish Type IV
Bass/Medaka
Flatfish Type I
Salmonid Type I
Mammal

Figure 17

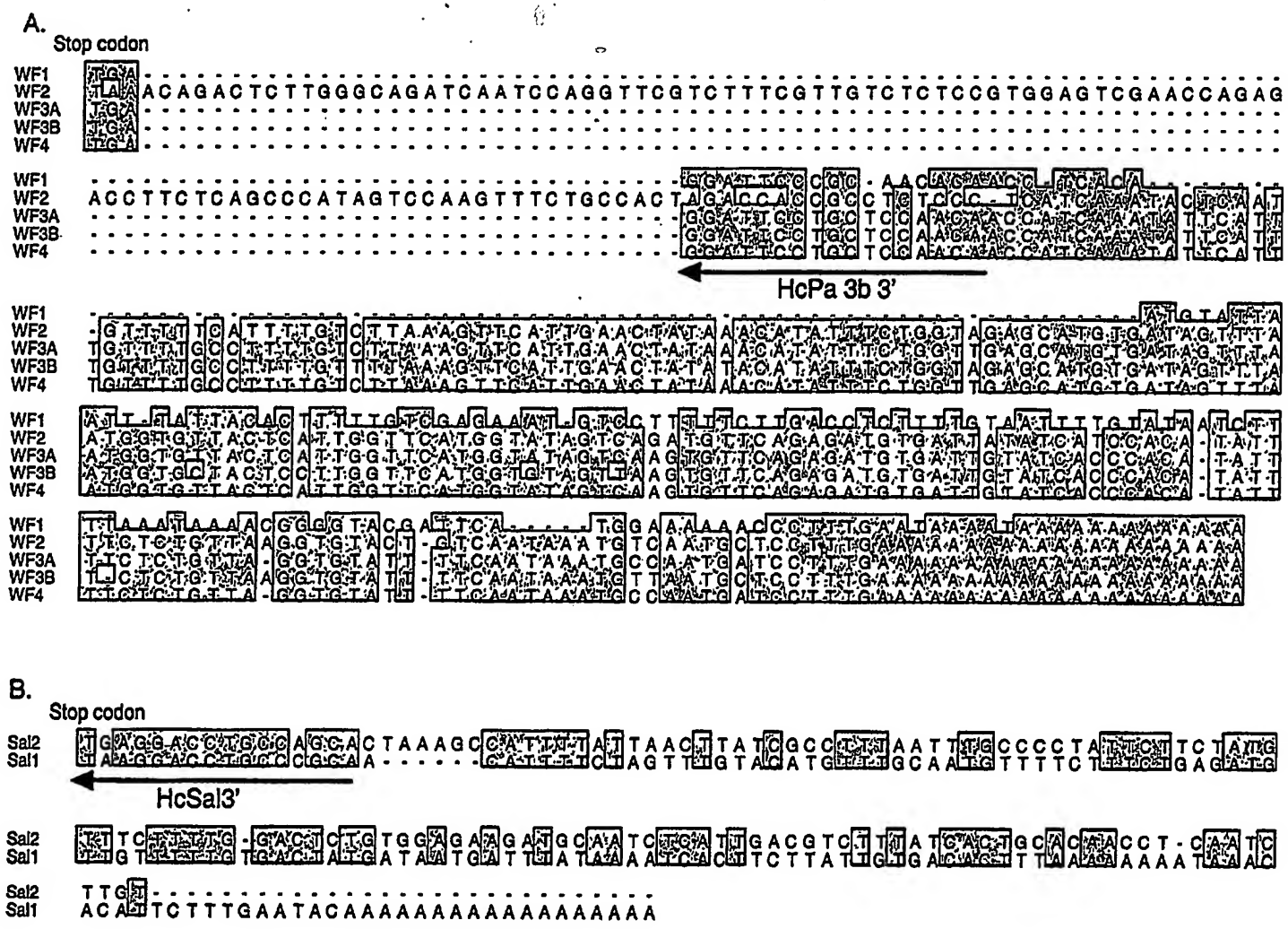

A.
Stop codon
WF1
WF2
WF3A
WF3B
WF4
HcPa 3b 3'
B.
Stop codon
Sal2
Sal1
HcSal3'

Figure 18

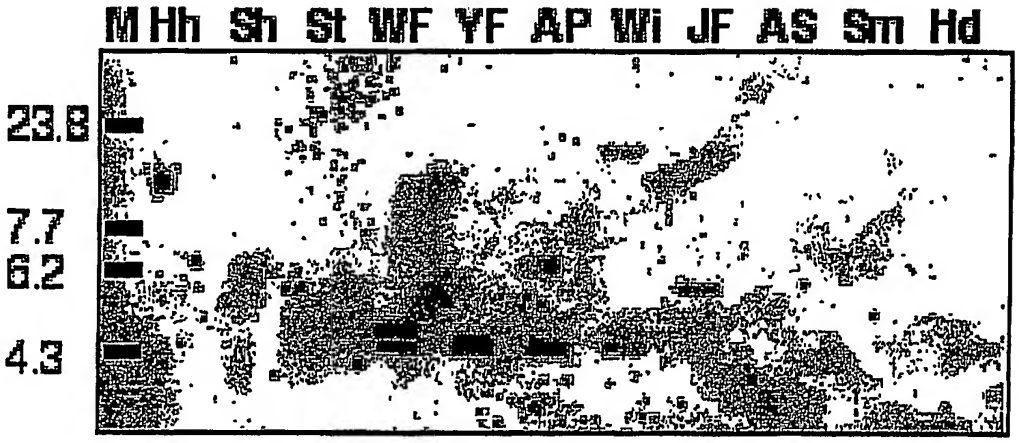


Figure 19

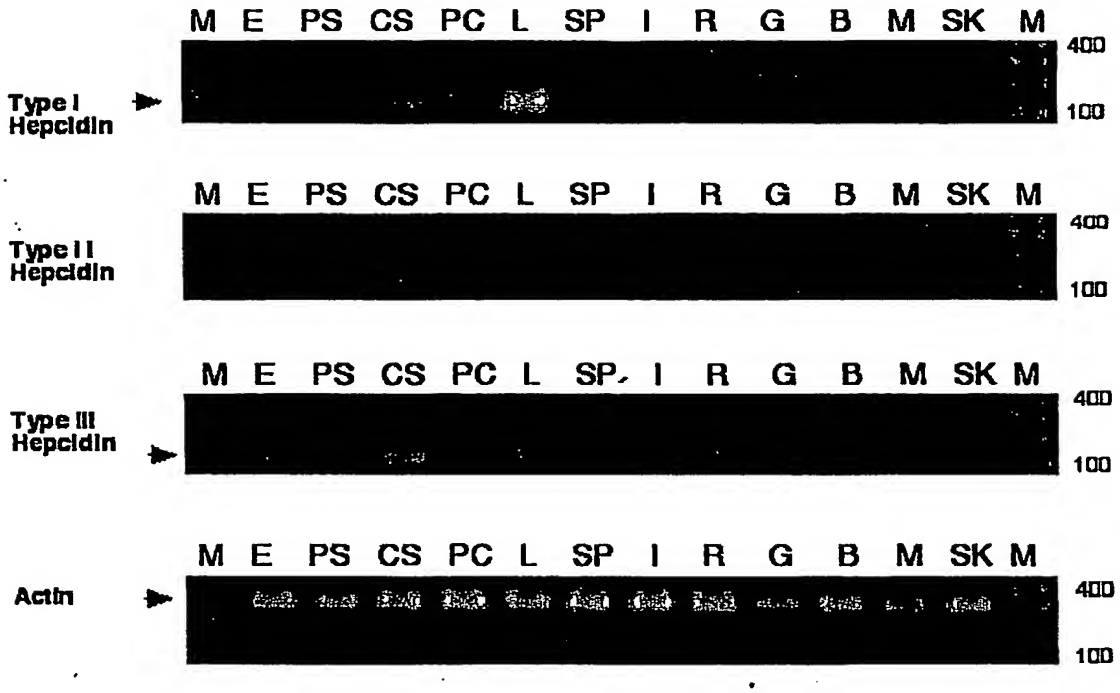

M E PS CS PC L SP I R G B M SK M
400
100
Type I Hepcidin
M E PS CS PC L SP I R G B M SK M
400
100
Type II Hepcidin
M E PS CS PC L SP I R G B M SK M
400
100
Type III Hepcidin
M E PS CS PC L SP I R G B M SK M
400
100
Actin

Figure 20

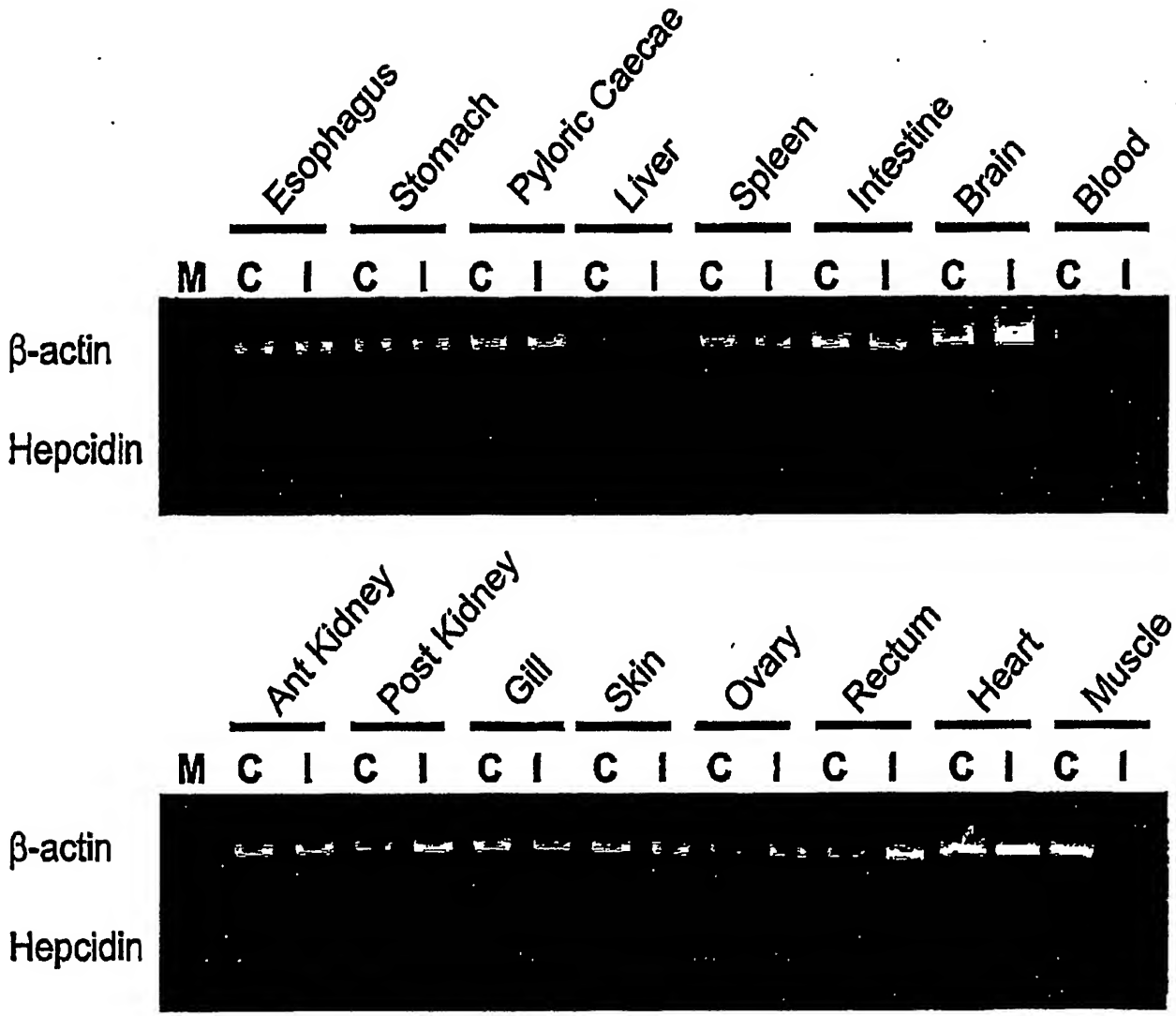

Esophagus
Stomach
Pyloric Caecae
Liver
Spleen
Intestine
Brain
Blood
M C I C I C I C I C I C I C I C I
β-actin
Hepcidin
Ant Kidney
Post Kidney
Gill
Skin
Ovary
Rectum
Heart
Muscle
M C I C I C I C I C I C I C I C I
β-actin
Hepcidin

Fig. 21A

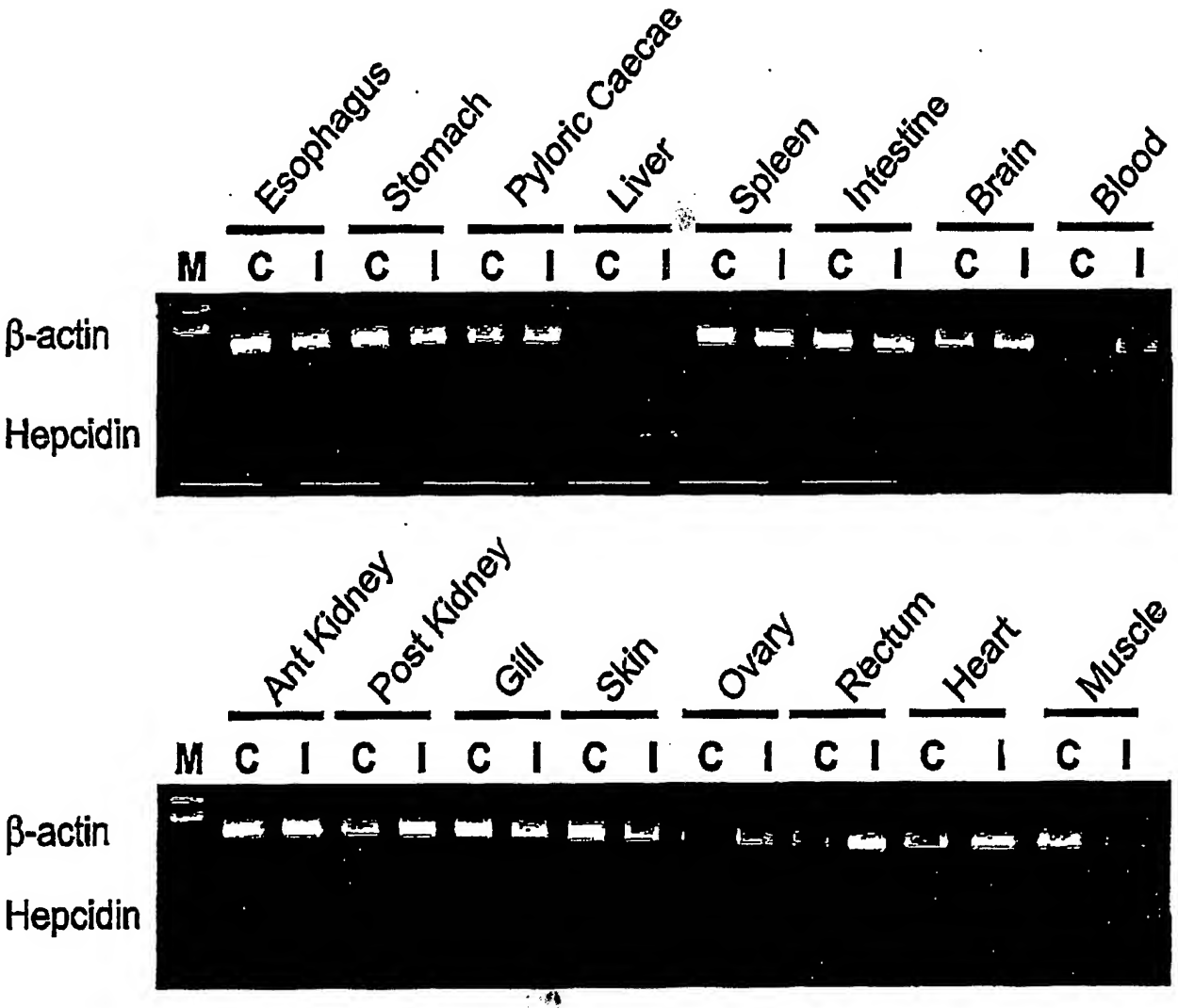

Esophagus
Stomach
Pyloric Caecae
Liver
Spleen
Intestine
Brain
Blood
M C I C I C I C I C I C I C I C I
β-actin
Hepcidin
Ant Kidney
Post Kidney
Gill
Skin
Ovary
Rectum
Heart
Muscle
M C I C I C I C I C I C I C I C I
β-actin
Hepcidin

Fig. 21B

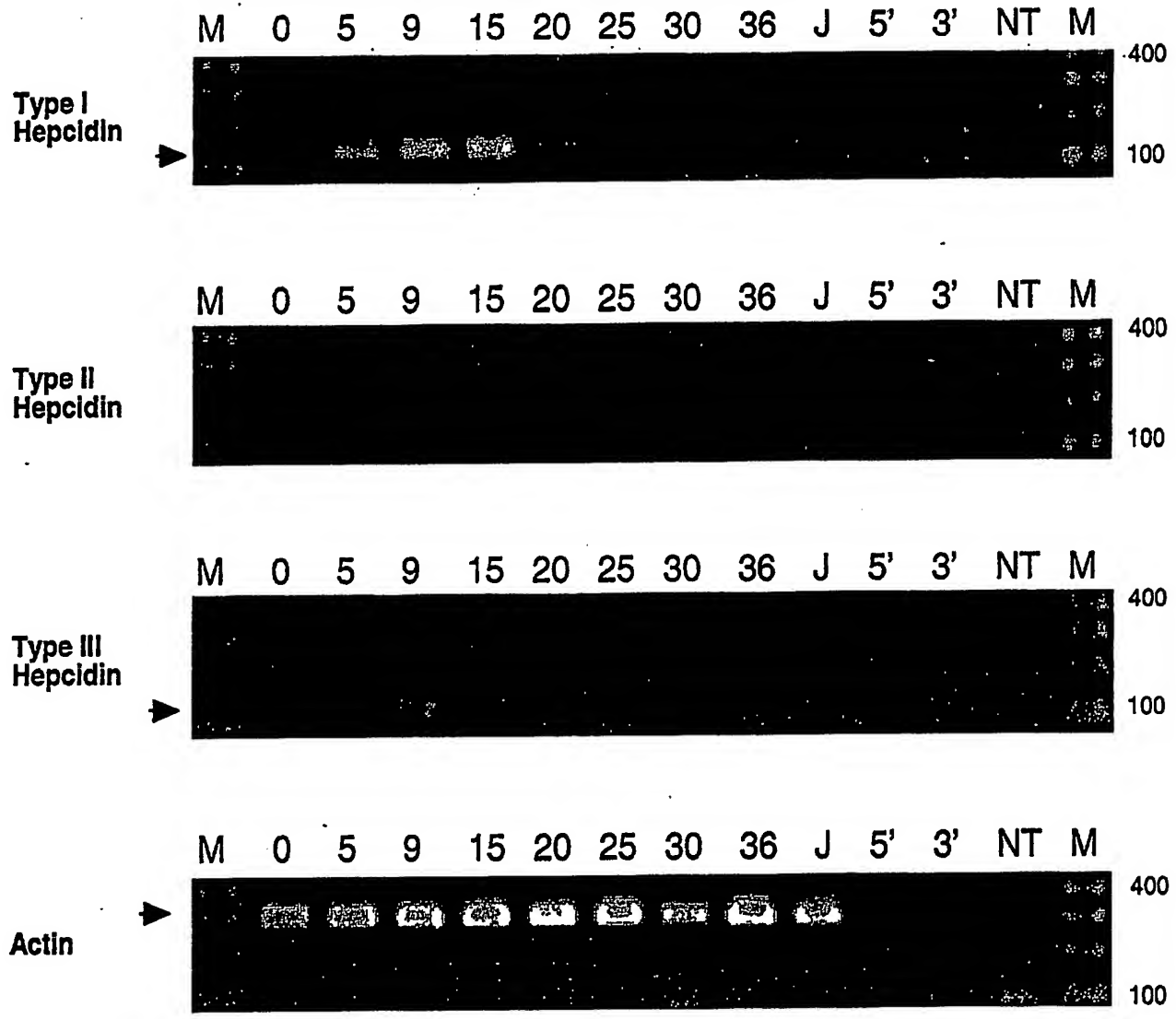

M 0 5 9 15 20 25 30 36 J 5' 3' NT M
Type I Hepcidin
400
100
M 0 5 9 15 20 25 30 36 J 5' 3' NT M
Type II Hepcidin
400
100
M 0 5 9 15 20 25 30 36 J 5' 3' NT M
Type III Hepcidin
400
100
M 0 5 9 15 20 25 30 36 J 5' 3' NT M
Actin
400
100

Figure 22

Table 1. Nucleotide sequences of oligonucleotides used for isolating pleurocidin-like sequences

Primer	Amino Acid Sequence	Nucleotide Sequence (5' ⇒ 3')
Screening cDNA library		
PleuroA	FFKKAAHVGKH	TTCTTCAAGAAGGCYGCYCAYGT[C/G]GG [C/A]AAGCA
PleuroB	HVGKAALTHYL[1]	CAYGT[C/G]GG[C/A]AAGGCYGCYCT[C/G] AA[C/T/A]CAYTACCT
Genomic PCR and RT-PCR		
PL1	5' untranslated	GCCCACTTTGTATTCGCAAG
PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
PL5'	MKFTATF	ATGAAGTTCACTGCCACCTTC
PL3'	KRAVDE[1]	TCATCGACTGCGCGCTT

[1]complement

Table 2. Nucleotide sequences of oligonucleotides used for assay of pleurocidin-like gene expression in different tissues and at different stages of development of winter flounder

Gene	Primer	Amino Acid Sequence	Nucleotide Sequence (5' ⇒ 3')
WF1	RTWF1	KGRWLER	AAGGGCAGGTGGTTGGAAAGG
	RTWF1/3'	YQEGEE[1]	CCCTCCCCCTCCTGGTA
WF1a	RTWF1a	RKRKWLR	CGTAAGAGAAAGTGGTTGAGA
	RTWF1a/3'	YQEGEE[1]	CCCTCCCCCTCCTGGTA
WF2	RTWF2	KAAHVG	AAGGCTGCTCACGTTGGC
	PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
WF3	RTWF3	FLGALIK	TTCTTAGGAGCCCTTATCAAA
	RTWF3/3'	YDEQQE[1]	CTCCTGCTGCTCGTCATA
WF4	RTWF4	HGRHAA	CATGGTCGTCATGCTGCC
	PL2	3' untranslated	CTGAAGGCTCCTTCAAGGCG
WFYT	RTWFYT	GFLFHG	GGGATTTCTTTTTCATGG
	RTWFYT/3'	SFDDNP[1]	GGGTTGTCATCGAATGAG
WFX	RTWFX	RSTEDI	CGTTCTACAGAGGACATC
	RTWFX/3'	DDDDSP[1]	GGGGCTGTCATCATCATC

Table 3. Nucleotide sequences of primers used in RT-PCR assays to analyse hepcidin gene expression. The amino acid sequence on which the 5' primer was based is shown. The 3' primers were within the 3' untranslated region (3' UTR). The annealing temperatures used in the PCR reactions and the sizes of the amplification products are listed.

Type (size)	Primer	Amino acid aequence	Nucleotide sequence (5'⇒3')	Annealing temperature	Product size (bp)
Winter flounder					
Type I	HcPA1 5' HcPA1b 3'	WMENPT 3'UTR	TGGATGGAGAATCCCACC GTGAGGTTGTGTTGCGGG	50°C	137
Type II	HcPA2 5' HcPA2b 3'	GMMPNN 3' UTR	GGGATGATGCCAAACAAC ACTTGGACTATGGGCTGAG	50°C	180
Type III	HcPA3 5' HcPA3b 3'	WMMPNN 3' UTR	TGGATGATGCCATACAAC GTTGTTGGAGCAGGAATCC	50°C	118
Actin	ActF (WF) ActR (WF)*	AALVVD VLLTEAP*	TCGCTGCCCTCGTTGTTGAC GGAGCCTCGGTCAGCAGGA	50°C	312
	ActinF1 Actin R1	VFPSIV HTFYNEL	GTGTTCCATCCATCGTC GAGCTCGTTGTAGAAGGTGT	50°C	194
Atlantic salmon					
Type I	HCSS 5' Hep Liv R	MHLPEP 3' UTR	ATGCATCTGCCGGAGCCT CATTGCAAACATGTACAAACTAG	55°C	163
Type II	Hep Sp F Hep Sp R	MNLPMH 3' UTR	ATGAATCTGCCGATGCA GGGCAAATTAAAGGCG	52°C	163
Actin	Act400F Act400R	IVGRPRHQ GYALPHAI	TCGTCGGTCGTCCCAGGCATCAG ATGGCGTGGGGCAGAGCGTAACC	52°C	400

* complement

Table 4. Sequences of pleurocidin-like peptides used for activity testing. Final peptide sequences and patterns of C-terminal amidation were selected based on the analysis of translated nucleotide sequences and on principles described in the text.

Origin	Amino acid sequence
Winter Flounder (1)	GKGRWLERIGKAGGIIIGGALDHL-NH_2
Winter Flounder (1a)	WLRRIGKGVKIIGGAALDHL-NH_2
Winter Flounder (1a-l)	GRRKRKWLRRIGKGVKIIGGAALDHL-NH_2
Winter Flounder (2) 2.1	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2
Winter Flounder (3)	FLGALIKGAIHGGRFIHGMIQNHH-NH_2
Winter Flounder (4) 1.1	GWGSIFKHGRHAAKHIGHAAVNHYL-NH_2
Yellowtail Flounder YT2	RWGKWFKKATHVGKHVGKAALTAYL-NH_2
Winter Flounder X	RSTEDIIKSISGGGFLNAMNA-NH_2
Winter Flounder Y	FFRLLFHGVHHGGGYLNAA-NH_2
Winter Flounder Z	FFRLLFHGVHHVGKIKPRA-NH_2
American Plaice AP1	GWKSVFRKAKKVGKTVGGLALDHYL-NH_2
American Plaice AP2	GWKKWFNRAKKVGKTVGGLAVDHYL-NH_2
American Plaice AP3	GWRTLLKKAEVKTVGKLALKHYL-NH_2
Witch Flounder GcSc4C5	AGWGSIFKHIFKAGKFIHGAIQAHND-NH_2
Witch Flounder GcSc4B7	GFWGKLFKLGLHGIGLLHLHL-NH_2
Witch Flounder GC3.8-t	GWKKWLRKGAKHLGQAAIK-NH_2
Witch Flounder GC3.8	GWKKWLRKGAKHLGQAAIKGLAS
Witch Flounder GC3.2	GWKKWFTKGERLSQRHFA
Halibut Hb26	FLGLLFHGVHHVGKWIHGLIHGHH-NH_2
Halibut Hb18	GFLGILFHGVHHGRKKALHMNSERRS

[a] Peptide predicted from expressed tag and/or expression confirmed by RT-PCR and/or by *in situ* hybridization.
[b] Peptide predicted from genomic sequence
[c] Pseudogenes
[d] NRC-2 and NRC-3 are both derived from the same sequences with the latter including an additional N-terminal fragment.

Table 4a. Bacterial and *Candida* strains used in this study.

Species	Code ID	Comment
Escherichia coli	C498, UB1005	Parent of DC2
Escherichia coli	C500, DC2	Outer membrane-permeable mutan
Escherichia coli	C786, CGSC4908	Triple auxotroph (thy, uri, L-his)
Salmonella enterica s. Typhimurium	C587, 14028S	Parent of C610
Salmonella enterica s. Typhimurium	C610, MS4252S	Supersusceptible strain
Pseudomonas aeruginosa	H187, K799	Parent of H188
Pseudomonas aeruginosa	H188, Z61	Supersusceptible strain
Enterococcus faecalis	C625, ATCC29212	Standard strain (ATCC)
Staphylococcus aureus	C622, ATCC25923	Standard strain (ATCC)
Staphylococcus aureus	C623, SAP017	MRSA clinical isolate (from Tony
Staphylococcus epidermidis	C960, ATCC14990	Standard strain (ATCC)
Staphylococcus epidermidis	C621	Clinical isolate (from David Speert
Bacillus subtilis	C971, ATCC6633	Standard strain (ATCC)
Aeromonsa salmonicida	99-1, A449	Field isolate being sequenced at IM
Aeromonas salmonicida	97-4	Field isolate
Candida albicans	C627, CALB105	Yeast test strain

Table 5. Sizes of introns (in bp) in genomic sequences amplified using primers PL5' and PL3'

Gene	Exon 1	Intron 1	Exon 2	Intron 2	Exon3	Total
WF1	154	539	31	95	82	901
WF1a[1]	103	?	31	?	82	?
WF2[2]	100	525	31	108	49	813
WF3	100	374	19	97	64	654
WF4[2]	100	230	31	101	49	511

[1]Intron sizes could not be determined as this sequence is only represented by an RT-PCR product

[2]Sequences were also amplified using primer PL1 and PL2

Table 6. RT-PCR products from skin and intestine corresponding to different pleurocidin genes

Skin	Intestine	Size	Band
4	n/d[1]	265bp	WF1
5	2	175bp	WF2
4	9	175bp	WF3
n/d[1]	n/d[1]	-	WF4
n/d[1]	7	215bp	n/d[2]

[1] not detected
[2] not detected by genomic PCR (corresponds to WF1a)

Table 7. Sizes of bands (in kb) hybridising to pleurocidin probes in *Bam*HI and *Sst*I digests of winter flounder DNA

Probe	*Bam*HI	*Sst*I
WF1	>24, 6	19, 17, 4.5, 4.4, 3.0, 2.9, 2.2, 1.3, x
WF2	6	19, 17, 4.5, 4.4, 2.9, x 1.3, x
WF3	>24	19, 17, 4.5, x 2.9, x 2.2, 1.3, x
WF4	17, 6	19, 17, 4.5, 4.4, 2.9, x 2.2, 1.3, 1.2

x=no hybridising band evident

Table 8. Minimal inhibitory concentrations of pleurocidin-like cationic antimicrobial peptides against a wide spectrum of bacterial pathogen and *Candida albicans*. Pathogens were grown in

	A.sal 99-1	*A.sal* 97-4	*S.typh* MS4252 s	*S.typh* 14028s	*P.aeru* K799	*P.aeru* Z61	*E.coli* C786	*E.coli* UB1005	*E.coli* DC2	*S.epi* C621
NRC-1	64	64	16	>64	>64	32	32	32	32	>64
NRC-2	>128	128	64	>64	64	32	64	64	64	>64
NRC-3	2	4	2	8	2	1	2	8	2	8
NRC-4	2	2	2	16	8	4	2	4	2	8
NRC-5	>64	>64	64	>64	>64	32	64	64	>64	32
NRC-6	4	4	4	64	16	4	4	4	2	>64
NRC-7	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
NRC-8	>64	>64	>64	>64	>64	>64	>64	>64	>64	>64
NRC-9	>64	>64	64	>64	>64	64	64	>64	>64	>64
NRC-10	>64	32	16	>64	32	8	32	32	32	32
NRC-11	8	8	4	32	32	4	4	16	4	64
NRC-12	2	2	2	8	4	1	2	8	2	8
NRC-13	4	2	2	8	4	1	2	4	2	4
NRC-14	32	16	16	>64	32	8	16	16	16	16
NRC-15	8	16	4	16	8	4	8	8	8	4
NRC-16	2	1	0.5	16	4	1	1	2	0.5	16
NRC-17	2	1	1	8	4	2	1	4	1	32
NRC-18	>64	128	32	>64	>64	64	64	64	64	>64
NRC-19	64	>64	16	64	32	8	32	16	32	8
NRC-20	>64	>64	>64	>64	>64	64	>64	>64	>64	>64

Mueller-Hinton broth and exposed to a range of concentrations of the specified peptide. The lowest peptide concentration which inhibited bacterial growth by at least 50% was recorded as the minimal inhibitory concentration.

Table 9. Characteristics of winter flounder and Atlantic salmon hepcidin-like peptides

Name	Total Amino Acids	Total Cysteines	Molecular Weight	pI
WF1	27	8	3066	8.75
WF2	19	6	1992	5.54
WF3	22	8	2367	8.74
WF4	22	8	2256	8.52
Hb5.3	22	8	2363	8.75
Sal8.6	22	8	2331	8.76
Hb17	22	8	2391	8.76
Hb1.1	22	8	2391	8.76
Hb357	22	5	2397	7.84
Hb7.5	25	8	2881	8.53
Sal2.1	25	7	2925	8.60
Sal1	25	8	2720	7.73
Sal2	25	8	2881	8.53

Table 10. Semi-quantitative RT-PCR analysis of hepcidin expression in Atlantic salmon during bacterial challenge

Tissue	Type I Hepcidin			Type II Hepcidin		
	Control	Infected	Ratio	Control	Infected	Ratio
Esophagus	nd	0.08	↑	nd	0.09	↑
Stomach	nd	0.09	↑	nd	0.27	↑↑
Pyloric caecae	nd	0.14	↑	nd	0.37	↑↑
Liver	1.19	2.36	2	nd	1.45	↑↑↑
Spleen	nd	0.18	↑	nd	0.41	↑↑
Intestine	nd	0.21	↑	nd	0.33	↑↑
Brain	nd	nd	0	nd	0.50	↑↑
Blood	0.82	0.84	1	nd	nd	
Anterior kidney	0.06	0.07	1.2	nd	0.08	↑
Posterior kidney	0.07	0.14	2	nd	0.11	↑
Gill	0.13	0.12	1	0.08	0.07	1
Skin	0.14	0.18	1.3	0.07	0.09	1.3
Ovary	nd	nd	0	nd	nd	0
Rectum	0.07	0.13	2	nd	0.08	↑
Heart	nd	nd	0	nd	0.43	↑↑
Muscle	0.38	0.8	2.1	nd	0.60	↑↑

Pixel densities obtained by densitometry are expressed relative to the actin signal. The ratio of infected:control was calculated where numerical values were obtained for both conditions. nd, not detected; ↑ weakly up-regulated; ↑↑ strongly up-regulated.

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☑ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☑ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☑ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.